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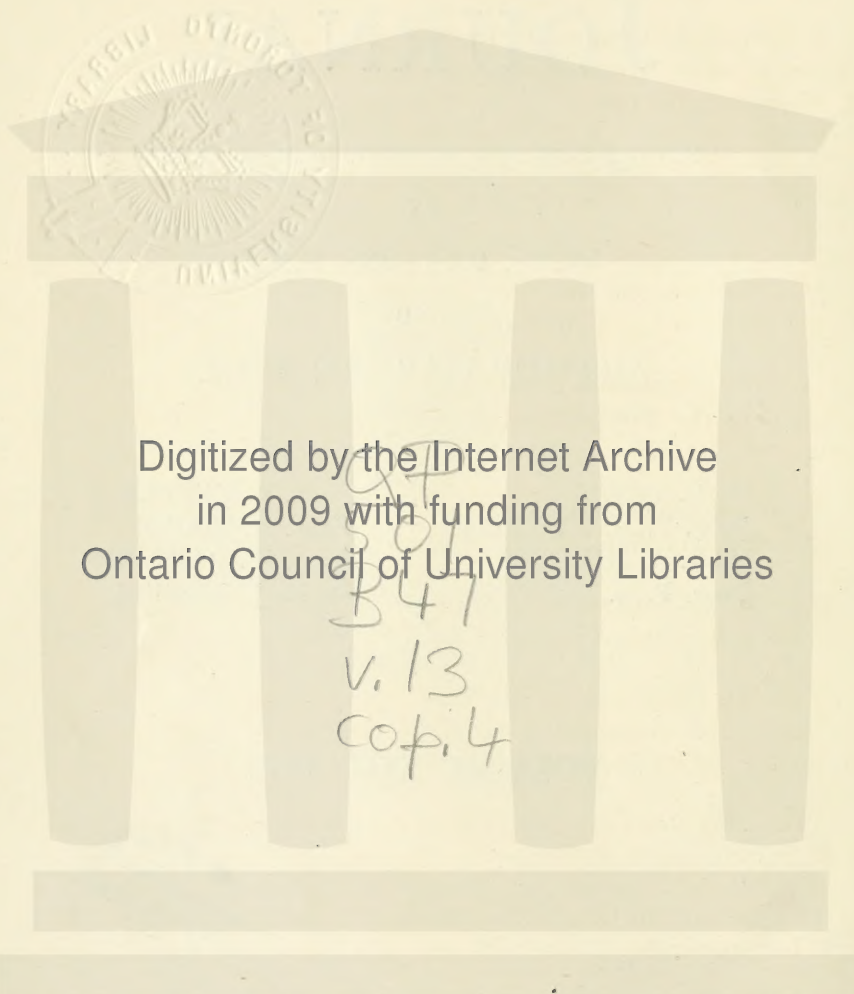
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CONTENTS

No. 1 (MAY)

	PAGE
I. Oxidising Enzymes. I. The Nature of the "Peroxide" naturally associated with certain direct Oxidising Systems in Plants. By M. WHELDALE ONSLOW	1
II. The Effects of Acids, Alkalies, and Sugars on the Growth and Indole Formation of <i>Bacillus coli</i> . By F. J. S. WYETH. (With One figure)	10
III. Observations on the Albuminoid Ammonia Test. By E. A. COOPER and J. A. HEWARD	25
IV. The Composition of Starch. Part I. Precipitation by Colloidal Iron. Part II. Precipitation by Iodine and Electrolytes. By J. MELLANBY	28
V. Observations on the Accuracy of Different Methods of Measuring small Volumes of Fluid. By F. W. ANDREWES	37
VI. On the Separation of Antitoxin and its Associated Proteins from Heat-denaturated Sera. By A. HOMER	45
VII. On the Increased Precipitability of Pseudoglobulin and of its Associated Antitoxin from Heat-denaturated Solutions. By A. HOMER. (With Three figures)	56
VIII. The Relation of Sugar Excretion to Diet in Glycosuria. By J. MELLANBY and C. R. BOX. (With Six figures)	65
IX. Note on the Rôle of the Antiscorbutic Factor in Nutrition. By J. C. DRUMMOND	77
X. Researches on the Fat-soluble Accessory Substance. I. Observations upon its Nature and Properties. By J. C. DRUMMOND	81
XI. Researches on the Fat-soluble Accessory Substance. II. Observations on its Rôle in Nutrition and Influence on Fat Metabolism. By J. C. DRUMMOND	95

No. 2 (JULY)

	PAGE
XII. Note on Xerophthalmia in Rats. By E. C. BULLEY	103
XIII. The Preparation of Silica Jelly for use as a Bacteriological Medium. By A. T. LEGG	107
XIV. Electrical Conductivity as a Measure of the Content of Electrolytes of Vegetable Saps. By D. HAYNES	111
XV. Contributions to the Study of the Vegetable Proteases. By E. R. FISHER	124
XVI. On the Estimation of Sugar in Blood. By H. MACLEAN. (With Two figures)	135
XVII. The Picric Acid Method for the Estimation of Sugar in Blood and a Comparison of this Method with that of MacLean. By O. L. VAUGHAN DE WESSELOW. (With One figure)	148
XVIII. Enterointoxication—its Causes and Treatment. By A. DISTASO and J. H. SUGDEN	153
XIX. The Action of Ultra-Violet Rays on the Accessory Food Factors. By S. S. ZILVA. (With Four figures)	164
XX. The Influence of Deficient Nutrition on the Production of Agglutinins, Complement and Amboceptor. By S. S. ZILVA. (With Eight figures)	172
XXI. The Nomenclature of Blood Pigment and its Derivatives. By W. D. HALLIBURTON and O. ROSENHEIM	195
XXII. The Anti-Scorbutic Value of Dry and Germinated Seeds. By H. CHICK and E. M. DELF. (With Five figures)	199
XXIII. The Effect of Alcohol on the Digestion of Fibrin and Caseinogen by Trypsin. By E. S. EDIE	219

No. 3 (NOVEMBER)

	PAGE
XXIV. Studies on Coagulation. I. On the Velocity of Gelation and Hydrolysis of Gelatin Sol. By R. SHŌJI. (With Three figures) . . .	227
XXV. Nitrogen Partition in the Urine of the Races in Singapore. By J. A. CAMPBELL	239
XXVI. Chemical Structure and Antigenic Specificity. A Comparison of the Crystalline Egg-albumins of the Hen and the Duck. By H. D. DAKIN and H. H. DALE. (With Four figures)	248
XXVII. The Rôle of the Plasma Proteins in Diffusion. By T. H. MILROY and J. F. DONEGAN. (With Four figures)	258
XXVIII. The Effect of Methods of Extraction on the Composition of Expressed Apple Juice, and a Determination of the Sampling Error of such Juices. By D. HAYNES and H. M. JUDD	272
XXIX. A Comparison between the Precipitation of Antitoxic Sera by Sodium Sulphate and by Ammonium Sulphate. By A. HOMER. (With Eight figures)	278
XXX. The Direct Replacement of Glycerol in Fats by Higher Polyhydric Alcohols. Part I. Interaction of Olein and Stearin with Mannitol. By A. LAPWORTH and L. K. PEARSON	296
XXXI. The Direct Replacement of Glycerol in Fats by Higher Polyhydric Alcohols. Part II. The value of Synthetic Mannitol Olive Oil as a Food. By W. D. HALLIBURTON, J. C. DRUMMOND and R. K. CANNAN	301
XXXII. Relative Anti-Scorbutic Value of Fresh, Dried and Heated Cow's Milk. By R. E. BARNES and E. M. HUME. (With Three figures)	306

No. 4 (DECEMBER)

	PAGE
XXXIII. On the Mechanism of Oxalic Acid Formation by <i>Aspergillus niger</i> . By H. RAISTRICK and A. B. CLARK	329
XXXIV. On the Self-Purification of Rivers and Streams. By the late A. E. COOPER; E. A. COOPER and J. A. HEWARD	345
XXXV. On the Digestibility of Cocoa Butter. Part I. By J. A. GARDNER and F. W. FOX	368
XXXVI. A New Method for Preparing Esters of Amino Acids. Composition of Caseinogen. By F. W. FOREMAN	378
XXXVII. On Amino-Acids. Part II. Hydroxyglutamic Acid. By H. D. DAKIN	398
XXXVIII. Studies in the Acetone Concentration in Blood, Urine, and Alveolar air: I. A Micromethod for the Estimation of Acetone in Blood, based on the Iodoform Method. By E. M. P. WIDMARK. (With Two figures)	430
XXXIX. Studies on the Cycloclastic Power of Bacteria. Part I. A Quantitative Study of the Aerobic Decomposition of Histidine by Bacteria. By H. RAISTRICK. (With Seven figures)	446
XL. Nitrogen Metabolism in <i>Saccharomyces cerevisiae</i> . By L. H. LAMPITT. (With Three figures)	459
XLI. The Relationship of Lecithin to the Growth Cycle in Crustacea. By J. H. PAUL and J. S. SHARPE. (With One figure)	487
INDEX	491

I. OXIDISING ENZYMES. I. THE NATURE OF THE "PEROXIDE" NATURALLY ASSOCIATED WITH CERTAIN DIRECT OXIDISING SYSTEMS IN PLANTS.

BY MURIEL WHELDALE ONSLOW.

From the Biochemical Laboratory, Cambridge.

(Received December 13th, 1918.)

THE following account records some experiments which throw additional light on the nature of the oxidising enzymes of plants. The dual nature of an oxidase, *i.e.* peroxide-peroxidase, and the fact that one component, the peroxidase, is an enzyme, has been established by previous workers. The resolution of the system into its component parts has also been effected to some extent.

The present work shows that the peroxide may arise by oxidation of an aromatic compound of a particular type of structure, the oxidation being activated by the peroxidase itself. It is also shown that the aromatic compound can be separated from the peroxidase by a purely chemical method, and that the two components can afterwards be reunited again. The peroxide once formed cooperates, in the usual way, with the peroxidase in carrying out further oxidation reactions such as are characteristic of "oxidases."

DIRECT AND INDIRECT OXIDATION AND THE GENERALLY ACCEPTED INTERPRETATION.

It has been known for a long time that the tissues of many plants discolour or turn brown on injury, such as bruising, *e.g.* Potato tuber, Apple fruit¹. The same phenomenon may be brought about in many cases by exposure of the tissues to chloroform vapour. A certain number of plants, on the other hand, do not show discoloration under similar treatment.

Further, it has been shown that the expressed juices, or water extracts, of plants which exhibit discoloration on injury, when added to guaiacum tincture immediately produce a blue colour. The juices, or water extracts, of plants which do not discolour, do not give a blue colour with guaiacum tincture until hydrogen peroxide has been added.

¹ The discoloration on injury has been found to be characteristic of many or all members of certain Natural Orders, notably the Umbelliferae, Labiatae, Compositae and others. In other cases, only a fraction of the genera of an order apparently exhibit the phenomenon. Finally, in some orders, *e.g.* Cruciferae, the phenomenon is rare or possibly entirely absent [Wheldale, 1911]

It has been assumed, on the evidence of many investigators, that the phenomenon of discoloration of tissues on injury is connected with the action of oxidising enzymes.

The hypothesis generally accepted in regard to oxidising enzymes is one which classifies these substances as direct and indirect. When either the juice, or water extract, of a tissue gives immediately a blue colour, *i.e.* an oxidation product, with guaiacum solution, the tissue is said to contain a direct oxidising enzyme, or oxidase. If, however, the blue colour only appears after addition of hydrogen peroxide, the tissue is said to contain an indirect oxidising enzyme, or peroxidase. It has been postulated that, in general, a system which turns guaiacum blue consists of a peroxide and a peroxidase: the peroxidase acts upon the peroxide and transfers oxygen in an active state to readily oxidisable substances, such as guaiacum. In the case of plant tissues which give the direct reaction, it has been suggested that some organic substance in the plant acts as a peroxide, while in tissues giving the indirect reaction, the peroxidase only is present, and the peroxide may be supplied artificially in the form of hydrogen peroxide.

The original conception of the hypothesis of oxidising enzymes, which we owe to Chodat and Bach, was rather more complex than that outlined in the foregoing paragraph. These authors regarded an oxidase as consisting of two components, a peroxidase (as described above) and an oxygenase. "Als Oxygenasen bezeichnen Chodat und Bach den supponierten eiweisshaltigen oder organischen Anteil der bisherigen Oxydasen, der als Peroxyd bildender Körper sich mit dem Luftsauerstoff addierend, sich mit ihm zu einem Körper der allgemeinen Formel $F \begin{smallmatrix} \diagup O \\ \diagdown O \end{smallmatrix}$ verbindet. Mit anderen Worten, es sind fermentartige Körper, die sich mit dem Sauerstoff der Luft zu einem Peroxyd verbinden können. Sie werden, wie andere fermentartige Körper, durch Hitze zerstört, durch starken Alkohol gefällt, können vergiftet und geschädigt werden. Sie unterscheiden sich von gewöhnlichen Peroxyden nur dadurch, dass sie wahrscheinlich hochmolekulare Körper sind." [Chodat, 1910.]

DEVELOPMENT OF THE ABOVE HYPOTHESIS SUGGESTED BY THE PRESENT WORK.

The following modification of the hypothesis of oxidising enzymes, or, at any rate, of a certain *class* of these substances, is suggested from experimental evidence to be described later.

In plants, which brown on injury and give the direct oxidase reaction, there is present a peroxidase and, in addition, some aromatic compound containing the dihydroxy grouping characteristic of catechol. On injury or autolysis, the peroxidase itself activates the oxidation of the aromatic compound, and the oxidised product constitutes a peroxide (of Chodat and Bach). The peroxide-peroxidase system so formed will then blue guaiacum tincture.

It would appear, moreover, that in the above-mentioned plants the peroxidase is always associated with compounds containing the "catechol" grouping.

It has been found possible to prevent the formation of the peroxide-peroxidase system by extracting the aromatic compound with alcohol, leaving the peroxidase in the tissue residue. The peroxidase can then only give the indirect reaction. On adding the extract of the aromatic substance to the peroxidase, the system can now be synthesised and will give the direct reaction.

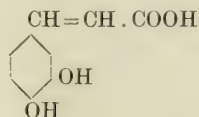
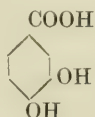
The above provides an extension of the system of Chodat and Bach, in that the peroxidase activates the formation of its own peroxide.

In plants, on the other hand, which give the indirect reaction and do not brown on injury, it appears that the peroxidase is not associated with compounds containing the "catechol" grouping, nor will these enzymes activate the oxidation of such compounds.

Aromatic compounds, such as those suggested, containing the dihydroxy grouping of the phenol catechol



are widely distributed in plants [corroborated by Wolff, 1917 and Wolff and Rouchelman, 1917]. Such substances may be, in some cases, protocatechuic acid, protocatechuic aldehyde, caffeic acid or derivatives of these:



There is evidence (based on the characteristic ferric chloride reaction) that plants, which brown on injury and give the direct reaction, do contain substances of this nature and that they are absent from plants giving only the indirect reaction.

It has been shown that the peroxidases of plants which give the direct reaction when added to solutions of catechol, protocatechuic and caffeic acids (on neutralisation), give brown oxidation products which are in effect peroxides. On addition of guaiacum to the mixture of the oxidised product and peroxidase, a blueing takes place. The same result is also obtained using the aromatic substance extracted from the plant itself.

The peroxidases of plants which give only the indirect reaction, on the other hand, do not act upon catechol, protocatechuic acid, etc., and no system blueing guaiacum is formed in their presence.

Hence it is to be pictured that, on injury or autolysis of tissues which discolour, the peroxidase comes into contact with a "catechol-like" substance whose oxidation it activates. Extracts or juices of the tissues will then blue guaiacum.

The conception of peroxidases, which oxidise polyphenols in the absence of hydrogen peroxide or other peroxide, differs from that formulated by Chodat and Bach. It might be maintained that the peroxidase used in the present account had traces of peroxide as impurity: even if this were so, the enzyme must still be defined as a peroxidase, since it is without action on guaiacum except in the presence of added hydrogen peroxide.

PREPARATION OF THE PEROXIDASE.

The employment of either expressed juices or aqueous extracts of tissues is not recommended for use in experiments on oxidising enzymes, since the presence of numerous other substances (sugars, tannins) largely interferes with the action of the enzymes. Moreover, in such liquids, on standing, reactions of many kinds may take place including those of reduction. Hence the following method of procedure has been employed, and either the fruit of the Pear or the tuber of the Potato may be used as material, though the method has been applied to other plants. The tissues are very rapidly pounded and extracted with cold alcohol, whereby the enzymes are precipitated and retained in the cell residue, while other substances, such as sugars and aromatics, are more or less completely removed.

The tissue of the Pear fruit constitutes a simpler case than that of the Potato tuber, since the latter contains, in addition to a peroxidase, a tyrosinase and a substance which it oxidises, presumably tyrosine. It is to the latter reaction that most of the darkening of expressed Potato juice is due. Tyrosinase, however, has no action on guaiacum [Chodat, 1910]. If Pear fruit is employed, it is advisable to select a variety which browns rapidly on injury, as the aromatic content, and possibly the activity of the peroxidase, may vary in different varieties.

For preparation of the peroxidase, a pear (or potato) is peeled, and from it are cut thin slices which are rapidly pounded in a mortar, after adding sufficient 96 % alcohol to prevent, as far as possible, any exposure of the tissue to air. The alcohol is then quickly filtered off on a filter pump. The residue can then be ground with more alcohol and filtered. The process should be repeated two or three times, and the grinding should be very thorough. After the final filtering, a white powder is left containing the insoluble residue of the cells including the peroxidase.

If a cold water extract of the residue be made and filtered, the following points can be demonstrated:

(a) It does not darken on standing in air. (A very slight darkening may take place after standing a number of hours.)

(b) Added to guaiacum tincture, it produces no blue colour within the time usually allowed for a positive reaction.

(c) Added to guaiacum tincture and hydrogen peroxide, a deep blue colour is obtained.

From the above results it appears that the alcohol has removed some part of the system responsible for darkening and for the direct reaction. This point will be considered again later (see section on extraction of aromatic substances).

Another point to be noted is that if the grinding is not sufficiently rapid, the residue may be discoloured, in which case the water extract of the residue may give some direct reaction. This will also be considered later (see section on laccase).

Similar results to the above were obtained with the tissues of fruits of the Apple and Greengage.

ACTION OF THE PEROXIDASE ON SOLUTIONS OF VARIOUS PHENOLS AND AROMATIC ACIDS.

If an aqueous solution of the Pear (or Potato) peroxidase (as prepared in the previous section) is added to a dilute solution of pure catechol, a yellowish tint is rapidly developed which eventually deepens to a yellowish-brown. It was also shown by performing the experiment in a closed vessel attached to a gas burette that there was an absorption of oxygen during the action of the peroxidase on catechol. If some of this oxidised solution, also containing peroxidase, is added to guaiacum tincture, the latter is immediately turned blue.

With a solution of protocatechuic acid, instead of catechol, the same result is obtained, but only if the protocatechuic acid is first neutralised.

Solutions of the peroxidase, together with guaiacum tincture, were also added to solutions of other phenols, *i.e.* phenol, quinol, resorcinol, pyrogallol and phloroglucinol, and to various acids, *i.e.* gallic, tannic, benzoic and salicylic, each acid being neutralised with sodium carbonate before the addition of the enzyme. The solution of pyrogallol turned brown, and there was some darkening with quinol, but when guaiacum was subsequently added, there was no appreciable blueing in any case.

It would thus appear that the Pear and Potato peroxidases are able to activate the oxidation of compounds containing the catechol nucleus, and that the oxidation products can act as peroxides as regards the oxidation of guaiacum.

The action of the Pear and Potato enzymes on a crude solution of caffeic acid was examined in the following way. Coffee berries contain caffeatannic acid which is said to be a glucoside of caffeic acid. The ground berries were extracted with hot water, filtered, and the filtrate precipitated with lead acetate. A yellow precipitate of the lead salt of caffeatannic acid separated out. This was decomposed with sulphuric acid, filtered from lead sulphate and neutralised. When the Pear peroxidase was added to this neutralised extract, followed by guaiacum tincture, a blue colour was obtained. It is to be supposed that the berry contains some free caffeic acid, since the caffeatannic acid has both hydroxyls replaced by sugar.

A similar browning of catechol solution, followed by blueing when guaiacum tincture is added, was obtained with enzymes from the following tissues (prepared in the way above described by treating with cold alcohol, and extracting the tissue residue with water): fruit of Apple and Greengage: leaf of Pear: flowers of Horse Chestnut (*Æsculus Hippocastanum*), and of a white variety of Foxglove (*Digitalis*). There is reason to believe that the enzymes of all plants which brown on injury and give the direct oxidase reaction (such as those above mentioned) will behave in the same way towards catechol and subsequently guaiacum.

The peroxidase from the fruit of Apple behaves like that of the Pear and Potato towards protocatechuic acid and crude caffeic acid, and also subsequently towards guaiacum.

On the other hand enzymes (by alcohol) from leaves of Yellow Alyssum, flowers of Mallow (*Malva moschata* var. *alba*), white Stock (*Matthiola*) and white Arabis were without action on catechol, and there was no blueing on subsequent addition of guaiacum tincture. The tissues of these plants give no browning on injury and no direct guaiacum reaction.

EXTRACTION OF AROMATIC SUBSTANCES.

This is carried out by cutting pears (or potatoes) into thin slices as rapidly as possible, and dropping the slices immediately into boiling 96 % alcohol. After boiling for a time, the hot alcohol is filtered off. The filtrate is then distilled *in vacuo* until all the alcohol is removed. The residue consists of a comparatively small bulk of a turbid aqueous solution of those substances which have been extracted by alcohol, the water being largely derived from the tissue of the fruit or tuber. To this extract, after filtering, a concentrated solution of lead acetate is added. A pale yellow precipitate is formed, and the acetate is added until no more precipitate is produced. The latter is filtered off and washed. It is then decomposed with the minimum amount of sulphuric acid, and the lead sulphate filtered off.

A portion of this filtrate is nearly neutralised (to litmus) with caustic potash solution, and with it the following tests are made:

(a) On addition of a few drops of 4 % ferric chloride solution, a green colour is produced. On further addition of a little dilute sodium carbonate, the green colour changes to bluish-purple and finally to purplish-red.

(b) An aqueous extract of the Pear (or Potato) peroxidase (prepared by alcohol) is added. The solution of aromatics, which has only a yellowish-brown tint, rapidly darkens to a deeper brown on standing about an hour.

(c) On addition of guaiacum tincture to the solution in (b) at any time within an hour after the addition of the peroxidase, a blue colour is obtained.

On addition of guaiacum tincture to the solution of aromatics which has been allowed to stand for some time without the presence of peroxidase, no blue colour is obtained within the time usually allowed for its appearance.

[The result in (a) is also given by extracts of the Apple and Greengage fruits and leaves of Pear, all of which brown on injury. There is no such result with extract of Alyssum leaves, which do not brown on injury.]

The remainder of the filtrate is then extracted with ether several times and the ether distilled off. The residue is taken up in water and the same test with ferric chloride repeated. It will be found to give the same reaction. It is not advisable to use the ether extract for testing with the oxidising enzyme, since ether itself may contain peroxides which complete the system.

From the above observations it is clear that hot alcohol will extract from the fruit (or tuber) a substance which is precipitated by lead acetate and is also soluble in ether. It gives a reaction with ferric chloride and sodium carbonate which is characteristic of catechol, protocatechuic acid, protocatechuic aldehyde and caffeic acid, all of which substances contain the ortho-dihydroxy grouping. This reaction will be referred to as the "catechol" reaction. It is also clear that the oxidation of the substance extracted in this way can be activated by the peroxidase, and the product of oxidation can function as a peroxide thus completing the system which oxidises guaiacum.

If the solution is neutralised before extraction with ether, the latter does not appear to extract the aromatic substance.

The precipitation by lead acetate, and the solubility in alcohol and ether, point to a resemblance between the aromatic substance of the Pear (or Potato) and both catechol and protocatechuic acid.

A similar and probably identical substance giving the "catechol" reaction was obtained by Reinke [1882] by hydrolysing Potato juice with hydrochloric acid and extracting with ether. It had been previously shown by Preusse [1879] that from a neutral or alkaline aqueous solution catechol can be extracted by ether, but not protocatechuic acid. Reinke obtained no substance giving the "catechol" reaction on ether extraction of the hydrolysed potato juice after neutralisation, and hence he concluded that the substance he described was not catechol. This conclusion appears to be confirmed by the experiments described in the present account. Reinke was also unable to identify his product with protocatechuic acid on account of its ready solubility in water, protocatechuic acid being only slightly soluble. Reinke suggests caffeic acid as the only alternative. The fact that the substance described in the present account is not extracted by ether from neutral aqueous solution indicates that it is probably an acid, but for complete identification it would be necessary to prepare it on a larger scale.

RELATIONSHIP BETWEEN THE PEROXIDASES OF THE PEAR AND POTATO AND LACCASE.

The direct oxidases of plants have generally been termed laccases by previous investigators [Chodat, 1910]. They are soluble in water, from which solution they can be precipitated by alcohol. Hence the method of preparation is usually by precipitation of the expressed plant juices by alcohol.

From results described in the present account it appears possible that many of the so-called laccases may be produced by the adsorption of a certain amount of the oxidised aromatic (produced during crushing and extraction) by the precipitate (by alcohol) containing the crude peroxidase. On redissolving in water, both peroxide and peroxidase will be present. It has been noted already that if the Pear (or Potato) tissue is either allowed to brown while pounding with alcohol, or is insufficiently extracted, the residue may be coloured, and in this case the water extract of the residue will give a direct guaiacum reaction.

It has been found that in the case of some tissues giving the direct reaction, it is practically impossible to prevent browning during maceration with alcohol, so that the residue is always somewhat coloured, and consequently the water extract gives the direct reaction.

INHIBITION OF OXIDISING ENZYMES.

It has been observed by several investigators that the action of oxidising enzymes may be inhibited by the presence of tannin, and, possibly, sugars. There is reason to believe that many plants may contain an enzyme of the type of that in the Pear fruit and Potato tuber, and also an aromatic with the catechol grouping, and yet, on injury, no browning takes place owing to inhibition by tannin present in the tissues.

CONCLUSIONS.

1. The tissues of many plants (*e.g.* fruit of Pear, tuber of Potato), on injury or exposure to chloroform vapour, turn brown: in other plants no browning occurs. Extracts of tissues which brown give the direct oxidase reaction with guaiacum: of those which do not brown, the indirect reaction only.

2. It would appear that the direct oxidase system in the Pear fruit and Potato tuber is due to the presence of a peroxidase (which blues guaiacum only on addition of hydrogen peroxide) and an aromatic substance giving the reaction characteristic of the catechol grouping. On injury, the peroxidase activates the oxidation of the aromatic with the formation of a peroxide. The peroxide-peroxidase system so formed will then blue guaiacum.

3. The aromatic substance giving rise to the peroxide can be extracted and separated from the peroxidase, thus preventing formation of the system. The system can be synthesised afterwards by combining the extracted aromatic and the enzyme.

4. There is evidence that in plants in general which brown on injury the peroxidase is associated with an aromatic substance giving the reaction characteristic of the catechol grouping. In such plants the peroxidases activate the oxidation of the aromatic substances (either obtained from their tissues or supplied from an artificial source) giving rise to peroxides, and the system

peroxide-peroxidase will then blue guaiacum. (In some plants however of this type the action may be masked or inhibited on injury by presence of tannins or other substances.)

5. Plants which do not brown on injury (and in which there is no apparent inhibition) do not contain a substance with the catechol grouping, and their enzymes do not catalyse the oxidation of substances with such a grouping.

This work is part of an investigation of oxidase action and fruit discoloration carried out for the Food Investigation Board under the general direction of Dr F. F. Blackman at the Biochemical Laboratory, Cambridge.

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II. THE EFFECTS OF ACIDS, ALKALIES, AND SUGARS ON THE GROWTH AND INDOLE FORMATION OF *BACILLUS COLI*.

BY FRANK JOHN SADLER, WYETH.

*From the Institute for the Study of Animal Nutrition, School of Agriculture,
Cambridge University.*

A Report to the Medical Research Committee.

(Received January 16th, 1919.)

THE EFFECT OF ALKALIES ON THE GROWTH OF *BACILLUS COLI*.

IN an earlier paper [Wyeth, 1918] it was shown that the final reaction produced by *B. coli* grown in 2 % glucose peptone was dependent upon the initial reaction of the medium, and was not a "physiological constant" as had been suggested by Michaelis and Marcora [1912].

The results obtained by the use of different media and acids were recorded for various initial reactions lying between $P_H = 4.23$ and absolute neutrality ($P_H = 7.00$). Since the introduction of the hydrogen electrode as a means of measuring the reaction (P_H) of liquids few results of the investigations of the growth of *B. coli* in media initially more alkaline than $P_H = 7.00$ have been recorded. It was thought desirable, therefore, to supplement the former research by making an examination of the behaviour of *B. coli* when grown in 2 % glucose peptone made alkaline by the addition of *N* sodium hydrate: an endeavour also being made to determine a "limiting value" of alkalinity, above which the growth of the organism is inhibited.

Material and Experimental Methods.

Pure cultures of *B. coli* obtained from human faeces were used. Experiments described in a former paper indicate that no differences in behaviour exist between strains of human and bovine origin. In this connection it may be noted that Murray [1916] made a comparative study of *B. coli* isolated from the faeces of man, horse, and cow. He agrees that all the strains prepared exhibited remarkable similarity of behaviour, and especially as regards their acid production.

A sterile 4 % glucose peptone medium was prepared by the method described in the former paper. It was then rendered alkaline by the addition

of *N* sodium hydroxide¹. If the alkali be added before sterilisation, caramelisation,—with consequent production of acids,—occurs and the medium does not attain the desired degree of alkalinity. The following procedure was therefore adopted. A number of flasks each containing 125 cc. of 4 % glucose peptone were prepared. To the contents of each flask the required volume, viz. $(125 - x)$ cc., of distilled water was added. The media were then sterilised by heating in an autoclave for 1 hour at 120°, allowed to cool, and finally the necessary volume (x cc.) of sterile *N* NaOH was added to the contents of each flask, which finally contained 250 cc. of sterile 2 % glucose peptone rendered alkaline with x cc. of *N* NaOH. In the series of experiments performed, the value of x was varied from 0 to 7.0 cc. of *N*/10 NaOH per 10 cc. of medium, and the initial reactions of the media ranged from $P_H = 7.0$ to $P_H = 11.0$. Inoculation was performed by adding to each 250 cc. of medium 0.5 cc. of a pure culture of *B. coli* grown for 18 hours in 2 % glucose peptone. The inoculated media were then incubated at 37° for 9 days, previous experiments having shown that the fermentation, as measured by change of final reaction (P_H) was practically complete at the end of the eighth day.

Results of Inoculation Experiments.

In the data given below H_1 , H_2 , etc. refer to strains of *B. coli* obtained from human faeces. Final P_H is the lowest value recorded during a period of 216 hours, and the + sign shown in column 5 of the table indicates that a positive result was obtained as regards fermentation, etc., while "0" indicates that a negative result was obtained.

Table I. *B. coli* grown in 2 % glucose peptone rendered alkaline by the addition of *N* NaOH.

Strain of <i>B. coli</i> used, H_4 .						
Time of incubation at 37°, 216 hours.						
Temperature of experiment, 20°.						
No. of flask	Cc. of <i>N</i> /10 NaOH* per 10 cc. of medium	Initial reaction P_H	Final reaction P_H	Fermentation	Cc. of <i>N</i> /10 NH_3 produced per 10 cc. of medium	Cc. of <i>N</i> /10 volatile acids produced per 10 cc. medium
1	7.0	10.80	—	0	—	—
2	6.0	10.50	—	0	—	—
3	5.0	10.28	—	0	—	—
4	4.0	9.82	4.82	+	0.02	2.65
5	3.5	9.72	4.81	+	0.03	2.78
6	3.0	9.51	4.81	+	nil	2.80
7	2.5	9.40	4.79	+	nil	2.68
8	2.0	8.97	4.72	+	nil	2.65
9	1.0	8.51	4.64	+	0.02	2.75

* Equivalent volumes of *N* NaOH were used.

¹ *N* NaOH was used since a higher P_H was required than could have been obtained by the use of *N*/10 NaOH, the approximate reaction of which is $P_H = 10.00$. For ease of comparison the equivalent amounts of *N*/10 NaOH are shown in the tables.

Consideration of Experimental Data.

The above results are typical of a large number obtained in several series of experiments, and of these the final P_H values are represented graphically in Fig. 1, Curve I. It is obvious that, as in the case of *B. coli* grown in a 2 % glucose peptone medium possessing an initial reaction less than $P_H = 7.0$, the final reaction produced by the organism when grown in alkaline glucose peptone (*i.e.* whose initial reaction is more alkaline than $P_H = 7.0$) is dependent upon the initial reaction (P_H) of the medium, although it varies within narrow limits. It was found that growth is permitted by a solution the initial reaction of which is $P_H = 9.82$, but that a medium of initial reaction $P_H = 10.28$ inhibits growth. There is therefore a "limiting value" for the initial reaction of alkaline glucose peptone which lies somewhere between these two values. The final acid reaction reached by a culture of *B. coli* in alkaline glucose peptone of the highest permissible degree of alkalinity (*i.e.* initial reaction $P_H = 9.82$) is such that its $P_H = 4.82$ which is identical with that determined by Clark [1915] as the final acid reaction of "human" *B. coli* grown in a medium containing 1 % Witte peptone + 1 % glucose.

In the "reaction resultant" curve for *B. coli* in 2 % glucose peptone shown in Fig. 1 (I) the initial reactions plotted on the 120° axis are so chosen as to cover the whole range of acidity and alkalinity within which growth of the organism in this medium is permitted. The form of the curve representing the final reactions (P_H) recorded shows that they form an ordered series lying between the acid limit of (approximately) $P_H = 4.27$, and the alkaline limit of (approximately) $P_H = 4.82$. Further, the connection existing between the initial reaction of the medium and the final reaction of the resulting culture is such that a change in the reaction of the former, whether it be in the acid or in the alkaline direction, produces a corresponding, but much smaller, change in the latter. The form of the reaction resultant curve in Fig. 1 (I) shows also that the resultant reactions obtained throughout the whole range of bacterial activity are due to the production of constant amounts of acid. In addition to measuring the final reactions of the cultures a quantitative determination of the principal products of fermentation was made, some of the results of which are shown in Table I, columns 6 and 7. It was found that practically no ammonia is formed during the fermentation, and that, although considerable volumes of volatile and fixed acids are formed, the actual amounts of each are approximately constant throughout the whole range of experiment, thus confirming the inference drawn from the form of the reaction resultant curve (Fig. 1 [I]). It is evident that the fermentation of *B. coli* in glucose peptone is to all intents and purposes exclusively saccharolytic. In order to find whether proteolysis supervened after saccharolytic fermentation was complete a number of cultures were allowed to ferment for more than 9 days. In a number of instances a rise of E.M.F. equal to 1-2 millivolts was observed, and this was sufficiently constant in occurrence to prohibit the assumption

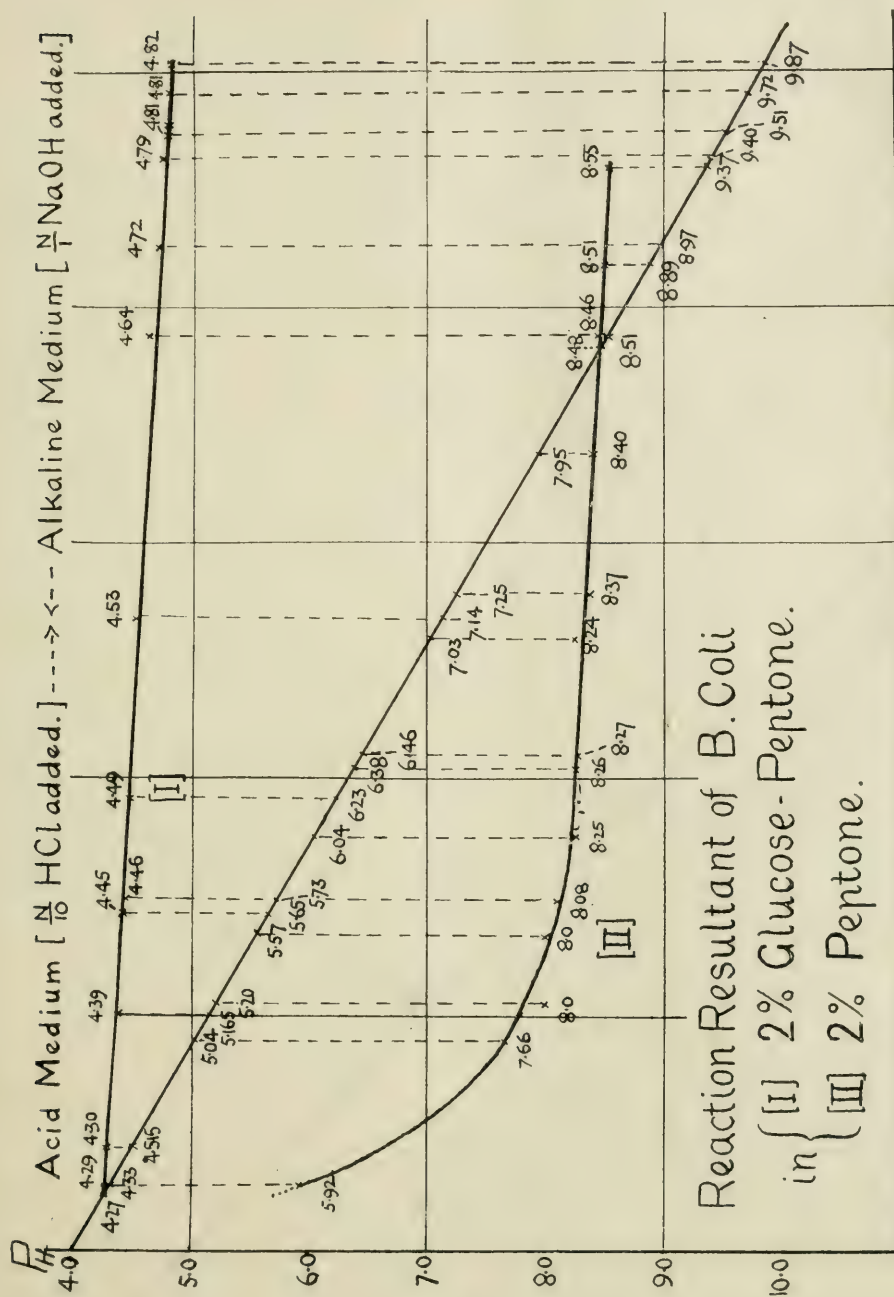


Fig. 1.

that it was always due to experimental error, although it must be conceded that such a variation in any individual culture certainly does fall within the limits of experimental error. That so small a reversion in the alkaline direction is found only in a certain proportion of the cultures proves conclusively that no appreciable amount of proteolysis occurs.

By constructing a titration curve for acetic acid in 2 % glucose peptone and using this in conjunction with the initial reactions of the cultures subjected to quantitative examination, and also with the amounts of acid formed therein, a curve was constructed showing what would be the reaction resultants theoretically produced by the formation of the volumes of acid actually found as a result of these experiments. This curve was of the same shape as that resulting from the final reactions (P_H) actually found, and given in Fig. 1 [1]. The conclusions arrived at after an examination of the initial and final reactions (P_H) of the cultures, and after a quantitative determination of the products of fermentation are therefore found to be in absolute agreement. It must be concluded that neither in the acid, nor in the alkaline range of initial reactions (P_H) is the final acid reaction attained by cultures of *B. coli* in 2 % glucose peptone a physiological constant. It has already been shown [Wyeth, 1918] that the degree of acidity necessary to inhibit the fermentation of *B. coli* in a given medium is subject to very slight variations when different acids are added to produce the initial acid reaction of the medium, and it is possible, therefore, that similar small variations of the alkaline inhibition-point may be caused by varying the alkali used to produce the initial alkaline reaction of the medium. Similar—but larger—variations may be expected to result from the use of different media, as has already been demonstrated for glucose peptone and glucose phthalate media whose initial reactions lie between P_H 4.0 and 7.0. It may be permissible, perhaps, to point out that in certain parts of the reaction resultant curve of *B. coli* grown in 2 % glucose peptone, the final reaction resultant values present so little variation that it is only by the comparison of a large number of cultures, the initial reactions (P_H) of all of which have been accurately measured by means of the H-electrode, that it is possible to detect this ordered relationship between the initial and final reactions of the cultures. As will be shown below, this applies to a less extent to the curve for cultures of the same organism in 2 % peptone. The very narrow limits between which the final reactions produced by *B. coli* grown in 2 % glucose peptone (or similar media) can vary, appears to have led a number of earlier investigators,—few or none of whom appear to have subjected the initial reactions of their media to small and accurately-measured variations,—to postulate a physiological constant for the final reaction of *B. coli* grown in the media with which they experimented.

The results recorded in an earlier paper, together with those now presented, conclusively disprove the existence of such a constant for *B. coli*, and Wolf and Harris [1917] arrive at the same conclusion as regards *B. sporogenes* and *B. perfringens*.

INDOLE PRODUCTION BY *B. COLI* GROWN IN 2 % PEPTONE.

Since the indole test is so frequently employed for diagnostic purposes, and as a proof of the presence of faecal *coli* in water and other media, it was thought desirable to determine whether indole production ran parallel with the growth of the organism, as measured by the change of P_H occurring in the medium employed, or whether fermentation unaccompanied by indole formation could be demonstrated.

The influence exerted by acids and carbohydrates upon the production of indole in cultures of *B. coli* has already engaged the attention of a number of investigators, but few exact measurements of the true acidity of such cultures appear to have been made. Although the primary object of the experiments described below was to determine the effect, if any, of acids and alkalies upon indole production it was found impossible entirely to separate this question from the much wider one of the influence of carbohydrates upon indole formation, since, as will be shown, the statements made by certain investigators regarding this latter phenomenon are by no means concordant. It was therefore considered desirable to undertake a number of experiments involving the use of peptone containing various carbohydrates.

Peckham [1897] found that the indole production of *B. coli* may be taken as an approximate measure of the amount of protein digestion due to the organism.

Theobald-Smith [1897] found that on the third or fourth day of incubation *B. coli* cultures in bouillon gave the indole reaction when the acidity was equal to $N/100$ (by phenolphthalein), or when litmus was coloured faintly blue by the liquid, while with Dunham's solution a well-marked violet-red colour was developed at the end of three days. He suggests that *B. coli* and other facultative anaerobes can produce indole only when they are in contact with oxygen.

Marshall [1907] showed that *B. coli* grown for five days at 37° in peptonised beef broth containing 2.0 % lactose failed to produce indole.

Glenn [1911] added considerably to our knowledge of the influence of carbohydrates on the production of indole by *B. coli*. His chief results are shown below.

Medium	Gas production	% acid produced in 4 days	Indole production hours		
			24	48	72...216
1 % peptone	—	0	—	+	+...+
1 % peptone + 1 % glucose	+	2.2	—	—	—...—
1 % peptone + 1 % saccharose	+	1.2	—	—	—...—
1 % peptone + 1 % lactose	+	2.0	—	—	—...—
1 % peptone + 1 % mannitol	+	2.0	—	—	—...—
1 % peptone + 1 % starch	—	0	+	+	+...+

He found that, in the case of *B. coli*, both glucose and lactose inhibit the production of indole, but, with *Proteus vulgaris*, lactose does not exert an inhibitory effect. He considers that his results "do not disprove the conclusion that the production of acid inhibits the formation of indole," and he concludes

also that the presence of 1 % or more than 1 % of glucose always inhibits the production of indole by *B. coli* grown in peptone¹. With *Proteus vulgaris* he found that the addition of lactic acid sufficient to produce an acidity equal to that of 0.5 % lactic acid inhibits indole production but that less than 0.5 % of the acid does not produce an appreciable retardation of the process.

Fischer [1915] found, however, that of the sugars, lactose, maltose, galactose, fructose, and glucose, only the last named completely retards the formation of indole. Total inhibition occurs after 43 hours in a medium containing from 1.80 to 2.25 % of glucose. He considers that the acids formed play no role in causing the retardation and that neither the H-ion concentration nor the concentration of the undissociated acids can be taken as the reason of the retardation. He concludes that the acid curves he constructed as well as the retardation experiments he performed with a mixture of galactose and glucose, make it appear possible that lactose is *not* first hydrolysed by *B. coli* but is fermented as such. The cause of the retardation, he suggests, depends upon a peculiar property of the glucose which enables it to inactivate the proteolytic enzyme produced by the *B. coli*.

Homer [1916] suggests that the lessened indole formation resulting from the activity of *B. coli* in glucose-containing media is due to the formation of a glucose-tryptophan complex which is less easily attacked than is tryptophan itself. This complex she regards as chemically unsuitable for bacterial decomposition and hence a lessened growth of *B. coli* ensues.

Zunz and György [1916] found it possible to grow *B. coli* in "l'eau physiologique" and in "l'eau physiologique + 1 % peptone" and that in these media indole formation occurred in less than 36 hours and 16 hours respectively. They conclude that indole production by *B. coli* depends upon the medium being rich in tryptophan and that the presence of certain carbohydrates inhibits the reaction. Unlike Glenn, they found that a medium containing peptone water + 1 % saccharose permits growth of *B. coli* together with indole formation within 96 hours—but not within 24 hours—after inoculation. Fischer does not appear to have experimented with saccharose, but, like Zunz and György—and unlike Glenn—he found that neither 1 % lactose nor 1 % maltose added to a peptone medium sufficed to inhibit the formation of indole by *B. coli*.

Material and Experimental Methods.

A number of media containing 2 % peptone were prepared and inoculated by methods similar to those previously described. The initial reactions of the tubed media were produced by the addition of either *N*/10 HCl or *N* NaOH in such quantities as to produce 20 different media whose initial reactions ranged from $P_H = 4.0$ to $P_H = 10.0$. In addition a number of tubes of 2 %

¹ Wolf has recently examined thirteen strains of *B. proteus* isolated from wounds, and has found that none of them produced indole. It is possible that Glenn was working with impure strains of this organism.

peptone of approximate neutral initial reaction ($P_H = 7.0$) were prepared each containing 2 % of one of the following carbohydrates: glucose, maltose, lactose, saccharose, starch and of mannitol.

In order to determine the influence, if any, exerted by acids or alkalies upon indole formation in 2 % peptone a number of sets of the tubes of this medium first mentioned above were inoculated with *B. coli* and incubated at 37°. They were tested for the presence of indole and the P_H of the cultures recorded at the end of 0, 4, 8, 24 hours after inoculation and then at intervals of 24 hours to the end of the ninth day of incubation. The rosindole and vanillin-hydrochloric acid tests were employed in determining the presence or absence of indole in the cultures. A number of the results obtained are embodied in Tables II and III, and on p. 19. The *B. coli* used were of faecal origin, and no differences were exhibited between those of human and those of bovine origin.

The results obtained with a number of cultures of *B. coli* grown in 2 % peptone, containing no glucose but having been made of different initial reactions (P_H) may be summarised as follows:

(i) Whether the medium be rendered acid (initial reaction P_H less than 7.0) by the addition of *N*/10 HCl, or alkaline (initial reaction P_H greater than 7.0) by the addition of *N* NaOH, indole production always occurs if the initial reaction of the medium be such as permits fermentation to take place.

(ii) If the initial reaction (P_H) of the medium approximate to either the acid ($P_H = 4.30$) or the alkaline ($P_H = 9.37$) limiting value for the growth of *B. coli* in 2 % peptone, the indole formation is subject to well marked retardation, in some cases being delayed for 144 hours, whereas over the greater part of the range of the initial reactions employed indole is formed within 4 to 8 hours after inoculation.

(iii) No matter what may have been the initial reaction (P_H) of the medium, indole was never detected in the culture unless and until its reaction reached a value lying between $P_H = 4.70$ and $P_H = 9.20$. Thus in a culture, the initial reaction of which was $P_H = 4.33$ and of which the acidity ultimately fell to $P_H = 5.92$, the production of indole was not observed until the expiration of 144 hours after inoculation, the reaction of the culture then being in the neighbourhood of $P_H = 4.80$. Similarly, in a culture of initial reaction $P_H = 9.37$, the alkalinity of which eventually diminished to $P_H = 8.55$, indole was first detected 96 hours after inoculation when the reaction of the culture was found to be $P_H = 8.80$. On the other hand with cultures whose initial reactions lay between $P_H = 8.89$ and $P_H = 5.20$ indole invariably was found in less than 24 hours after inoculation.

In no case was the presence of skatole detected.

Consideration of Results of Experimental Data.

It would appear that the presence of indole in a culture may safely be taken as an indication of the presence of *B. coli* of faecal origin, but that its

production—like the growth of the organism itself—is retarded by the presence in the medium of an excess of free acid or alkali. The inhibition of indole formation may, however, be one result of a lowered vitality of the *B. coli* consequent upon the presence of this excessive amount of acid or alkali, and it may even be a precursor of the death of the organism, brought about by the action of these substances. The results of these experiments appear to confirm Fischer's statement that the acids and alkalies formed as a result of fermentation by *B. coli* do not cause an inhibition of indole production. On this point Glenn comes to no definite conclusion although he appears to lean towards the opinion that the inhibition of indole formation *may* be due to the presence of acids formed during fermentation.

In experiments with *B. coli* grown in 2 % peptone, had the indole formation occurred *only* in the most acid media of the series under examination it might be argued from a comparison of Curves I and II of Fig. 1 that the inhibitory effect produced by the addition of glucose to a peptone medium might be due solely to the production of acids as a result of saccharolytic fermentation. This would, at first sight, appear to be more probable since the final acid reactions produced by *B. coli* fermenting in 2 % glucose peptone lie between $P_H = 4.27$ and $P_H = 4.82$. Indole formation is completely inhibited in cultures of *B. coli* in 2 % glucose peptone throughout this range of reaction, and also in 2 % peptone media so long as their reactions remain more acid than $P_H = 4.70$. Since in the latter cultures the inhibition is obviously the result of added acid, and the two ranges of reaction are practically identical, there is at hand an obvious explanation of the inhibitory action due to the presence of glucose in the former class of cultures. This explanation, however, cannot be correct for the following reasons:

(i) At no period during the fermentation of a 2 % glucose peptone medium does *B. coli* produce indole even when in the initial stages of the process the reaction of the culture is much less acid than $P_H = 4.82$ or $P_H = 4.70$.

(ii) The retardation of indole formation by *B. coli* fermenting in 2 % peptone occurs not only when excess of acid is present but also when there is excess of alkali present in the medium. Inhibition persists so long as the reaction of the culture is more alkaline than $P_H = 9.20$. It appears probable therefore that the inhibition of indole formation in cultures of *B. coli* in peptone containing excess of either acid or alkali is due to a cause entirely different from that which operates when the inhibition results from the presence of glucose in the medium. In the former case the inhibition may be due to a lowered vitality of the organism, produced by the action of the free hydrogen or hydroxyl ions present, while in the latter instance the cause of the inhibition may be attributed to a peculiar property of the glucose, which enables it to inactivate the proteolytic enzyme of the *B. coli*.

THE INFLUENCE OF CERTAIN CARBOHYDRATES AND ALLIED COMPOUNDS
UPON THE PRODUCTION OF INDOLE IN PEPTONE MEDIA.

For these experiments the tubed media containing 2 % peptone together with either 1 % or 2 % of added carbohydrate were used (p. 16). The media were inoculated with different strains of *B. coli*. The cultures were incubated at 37° and examined at the end of periods of x hours as recorded in the sub-joined tables.

Medium inoculated with <i>B. coli</i>	Indole formation at the end of hours					Gas production
	$x = 4$	8	24	48	96	
2 % peptone + 1 % glucose	0	0	0	0	0	+
2 % peptone + 1 % lactose	0	0	+	+	+	+
2 % peptone + 1 % maltose	0	0	+	+	+	+
2 % peptone + 1 % mannitol	0	(+ ?)	+	+	+	+
2 % peptone + 1 % saccharose	0	0	0	(+ ?)	+	+
2 % peptone + 1 % starch	+	+	+	+	+	0

Medium inoculated with <i>B. coli</i>	Indole formation at hrs after inoculation					Gas production
	$x = 4$	8	24	48	96	
2 % peptone	+	+	+	+	+	0
2 % peptone + 2 % glucose	0	0	0	0	0	+
2 % peptone + 2 % lactose	0	0	0	0	(+ ?)	+
2 % peptone + 2 % maltose	0	0	0	0	(+ ?)	+
2 % peptone + 2 % mannitol	0	0	0	(+ ?)	+	+
2 % peptone + 2 % saccharose	0	0	0	0	(+ ?)	+
2 % peptone + 2 % starch	+	+	+	+	+	0

From the above results it appears that the retardation produced by either 1 % or 2 % glucose in 2 % peptone may be regarded as absolute. The same percentages of starch produce no retardation, while that produced by mannitol is comparatively slight. Lactose and maltose appear to possess less retarding power than does glucose since 1 % of maltose or lactose produces but little effect while 2 % of either of these sugars produces a retardation approximating to the absolute inhibition resulting from the action of glucose. Since the initial reaction of all these cultures approximated to $P_H = 7.03$ it may be concluded that where retardation was observed it could not be ascribed to the initial presence (or to the ultimate production) of alkali or of acid, but must be the result of some specific action of the added carbohydrate. It must also be noted that where partial retardation occurred, the production of indole was observed at precisely the time when,—if inhibition were due to resultant acidity,—it should begin to be inhibited.

THE EFFECTS OF ACIDS AND ALKALIES ON THE GROWTH OF *B. COLI*
IN 2 % PEPTONE.*Material and Experimental Methods.*

A number of flasks and tubes of 2 % peptone medium of various initial reactions were prepared as described above. As, in the case of the experiments with glucose peptone, the acid used was $N/10$ HCl and the alkali

employed was *N* NaOH. Some twenty sets of media whose initial reactions ranged from $P_H = 3.50$ to $P_H = 10.50$ were prepared. Inoculation and incubation were performed as in the preceding experiments.

The tubed cultures were examined for changes of reaction (P_H) and for the production of indole, while the flask cultures were submitted to the same tests, and in addition, a quantitative determination of the products of fermentation was made.

The results obtained in the two series of experiments are shown in the subjoined Tables II and III and a reaction resultant curve for *B. coli* grown in 2 % peptone was constructed, and is shown in Fig. 1 (II).

Table II. *B. coli* grown in 2 % peptone rendered acid by the addition of *N*/10 hydrochloric acid.

Strain of *B. coli* used, H_8 .

Time of Incubation at 37°, 216 hours.

Temperature of Experiment, 20°.

No. of flask	No. of cc. of <i>N</i> /10 HCl added per 10 cc. of medium	Initial reaction P_H	Final reaction P_H	Fermentation	Cc. of <i>N</i> /10 NH_3 produced per 10 cc. of medium	Cc. of <i>N</i> /10 volatile acids produced per 10 cc. of medium	Indole formation
1	3.0	3.54	—	0	—	—	0
2	2.5	3.93	—	0	—	—	0
3	2.0	4.33	5.92	+	0.64	0.49	+ at 144 hrs
4	1.0	5.20	8.00	+	1.84	1.12	{ + at 12 hrs + + at 72 hrs
5	0.5	6.46	8.27	+	2.52	1.37	{ + at 12 hrs + + at 24 hrs
6	0.0	7.25	8.37	+	3.00	1.60	+ + at 12 hrs

Table III. *B. coli* grown in 2 % peptone rendered alkaline by the addition of *N* NaOH.

Strain of *B. coli* used, H_8 .

Time of Incubation at 37°, 216 hours.

Temperature of Experiment, 20°.

No. of flask	Cc. of <i>N</i> /10 NaOH added per 10 cc. of medium*	Initial reaction P_H	Final reaction P_H	Fermentation	Cc. of <i>N</i> /10 NH_3 produced per 10 cc. of medium	Cc. of <i>N</i> /10 volatile acids produced per 10 cc. of medium	Indole formation
1	5.0	10.23	—	0	—	—	0
2	4.0	9.87	—	0	—	—	0
3	3.0	9.37	8.55	+	4.5	3.95	{ + at 96 hrs + + at 168 hrs
4	2.0	8.89	8.51	+	4.1	3.34	{ + at 24 hrs + + at 48 hrs
5	1.0	8.51	8.46	+	3.5	2.75	{ + at 12 hrs + + at 24 hrs
6	0.5	7.95	8.40	+	3.2	2.21	+ + at 12 hrs

* Equivalent volumes of *N* NaOH were used.

Consideration of Experimental Data.

The salient fact revealed by a study of the values of the initial and final reactions (P_H) recorded in Tables II and III (columns 3 and 4) is that there is for *B. coli* grown in 2 % peptone,—as for the same organism in 2 % glucose peptone,—an obvious connexion between the initial reaction of the medium and the final reaction of the culture. This is represented graphically by the reaction resultant curve (II) in Fig. 1. Next it is observed that there is an “acid” and an “alkaline limiting value” of the initial reactions between which growth is permitted but beyond which the activity of the organism is inhibited. The limiting value in the acid range of initial reactions lies between $P_H = 4.33$, which permits, and $P_H = 3.93$ which inhibits the fermentation of *B. coli* in 2 % peptone. The degree of alkalinity which serves to inhibit fermentation lies between an initial reaction of $P_H = 9.37$ which permits and $P_H = 9.87$ which inhibits the process. The activity of *B. coli* in 2 % peptone is thus found to be determined by almost the same initial conditions of acid and alkaline reaction as is the case with the same organism fermenting in 2 % glucose peptone.

There are however striking differences between the behaviour of *B. coli* when grown in the two media, as may be seen by comparing Table I with Tables II and III, and also Fig. 1 (I) and Fig. 1 (II).

In both media the final reactions attained by a culture of the bacillus occupy positions on an unbroken curve, and in both media a diminution of the initial acidity (P_H) of the medium results in a diminution of the resultant acid reaction of the culture but beyond these general resemblances the similarity does not extend.

Whereas the range of initial reactions within which growth is possible has been shown to be practically identical for *B. coli* grown in the two media there is a remarkable difference between the range of final reactions attained in them. In 2 % glucose peptone the final reactions vary between the very narrow limits of $P_H = 4.27$ and $P_H = 4.82$ but in 2 % peptone the final reactions produced by the organism are as widely separated as $P_H = 5.92$ and $P_H = 8.55$ while it is quite possible that further experiments may lower the former of these two values to $P_H = 5.70$, as will be seen by an inspection of Curve II, Fig. 1.

It is obvious that in the case of *B. coli* grown in 2 % peptone any suggestion of a “physiological constant” for the final reaction values cannot be entertained. It will be noted that the curve (Fig. 1 [II]) representing the reaction resultant of *B. coli* grown in 2 % peptone cuts the axis on which the initial reactions of the medium are plotted at a point representing a reaction of $P_H = 8.48$. It is evident that if it were possible to prepare a 2 % peptone medium of which the initial reaction were exactly $P_H = 8.48$ the fermentation of *B. coli* in this medium should be unaccompanied by any change of reaction. Several attempts to prepare a medium of this initial reaction were made, but

without success. The medium most closely approximating to that desired had an initial reaction of $P_H = 8.51$. In it, fermentation was very active, indole, ammonia, volatile and fixed acids being formed in large quantities while the final reaction became slightly less alkaline—the ultimate value being $P_H = 8.46$.

Passing from this point to the acid "death-point" of the organism it is observed that for cultures in a medium of which the initial reaction is acid (P_H less than 7.0) or possessing an alkalinity of less than $P_H = 8.48$, the activity of the bacillus results in a change of the reaction of the culture in an alkaline direction. The inference which naturally would be drawn from an inspection of this region of the reaction resultant curve, viz. that the fermentation of *B. coli* in 2 % peptone the initial reaction of which is less alkaline than $P_H = 8.48$ results in the formation of an excess of alkaline products, the reaction of the culture thereby becoming more alkaline than $P_H = 8.48$, is confirmed by the results of the quantitative examination of the products of bacterial activity (Tables II and III, cols. 6 and 7).

In the absence of sugar, the action of *B. coli* on peptone is frankly proteolytic, the higher nitrogenous complexes being decomposed, with the consequent formation of ammonia, acids and indole.

When, however, *B. coli* ferments an alkaline 2 % peptone, the initial reaction of which is more alkaline than $P_H = 8.48$ the activity of the organism results in a diminished alkaline reaction in the culture (*e.g.* a medium whose initial reaction is $P_H = 8.89$, reaches a final reaction of $P_H = 8.51$). Reference to the results of quantitative examination of the fermentation products shows that this diminished alkalinity is due to a rapid increase in the amount of acid produced. In general it may be observed that, as the initial reaction of the 2 % peptone in which *B. coli* ferments is varied from $P_H = 4.33$ to $P_H = 9.37$, the production of alkali is at first more marked than that of acid, while in the latest stages the increase of acid production is greater than that of alkali formation. This accounts for the "reversion" of the course of the reaction when the critical point $P_H = 8.48$ is passed. It must be emphasised that the reversion does not proceed far enough to interrupt the regular path of the reaction resultant curve (Fig. 1 [II]) since any given culture eventually reaches a final reaction which is more alkaline than that attained by the next lower (more acid) member of the series.

A complete and accurate estimation of the fixed acids produced in the reactions was not performed, but sufficient data were obtained to show that in the neighbourhood of the "alkaline limiting value" ($P_H = 9.37$) the total acids formed were in excess of the alkaline products, whereas in cultures the initial reactions of which were acid, neutral, or slightly alkaline (initial $P_H = 4.33$ to 8.00) the ammonia production was greater than the formation of acids. It is evident that the theoretical yield of product of a culture whose initial reaction is $P_H = 8.48$ (and is therefore constant during fermentation), should consist of equivalent quantities of acid and alkali. With the aid of

titration curves for acetic acid and ammonia added to alkaline and acid 2 % peptone respectively a curve was plotted which represented the effect produced by the addition to 2 % peptone of the amounts of ammonia and acids found in the quantitative determinations performed.

This was done by taking in turn the initial reaction of each culture and finding from the acetic and titration curve what would be the effect upon the initial reaction if the amount of acid actually found by experiment were added to the medium. To the new reaction value thus obtained is then applied the correction representing the effect produced by the amount of ammonia the presence of which had been determined by the experiment, this correction having been found from the ammonia titration curve. The final resultant obtained in each case represents the theoretical "reaction resultant" of the fermentation. The whole series of values thus obtained was plotted as a reaction resultant curve, which was found to be similar to, but not quite coincident with, that shown in Fig. 1 (II). This indicates that the curve representing the observed final reactions is such as would result had it been plotted from a series of final reaction resultant values produced by the formation of increasing amounts of acids and ammonia in a series of media of increasing alkaline reaction, the amounts of these acids and ammonia being of the same order as those found in the foregoing experiments.

SUMMARY.

1. Growth of *B. coli* is possible in peptone media of certain well-defined initial reactions, the limiting values of which are but slightly, if at all, affected by the presence or absence of sugars.

2. The approximate limits of initial reaction are $P_H = 4.27$ to 9.87 .

3. A change of the initial reaction of the medium results in a change, similar in direction, but smaller in magnitude, in the final reaction of the culture.

4. In the case of *B. coli* grown in 2 % glucose peptone, while the initial reactions of the media vary from $P_H = 4.30$ to $P_H = 9.82$ the final reactions attained vary only between the very narrow limits of $P_H = 4.27$ and 4.82 .

5. In the case of the organism grown in 2 % peptone, when the initial reaction of the medium is varied from $P_H = 4.30$ to $P_H = 9.37$, the final reactions of the cultures vary from $P_H = 5.92$ ($5.70?$) to $P_H = 8.55$.

6. The saccharolytic fermentation resulting from the growth of *B. coli* in 2 % glucose peptone renders the culture more acid than the original medium.

7. The proteolytic fermentation resulting from the growth of *B. coli* in 2 % peptone causes an increase of final alkalinity in the resulting culture unless the initial reaction lies between the alkaline limiting value ($P_H = 9.37$ and $P_H = 8.48$ in which case the final reaction of the culture is less alkaline than the initial reaction of the medium.

8. The saccharolytic fermentation of *B. coli* in 2 % glucose peptone media of different initial reaction produces approximately constant amounts of acids and no appreciable amount of ammonia.

9. The proteolytic fermentation of *B. coli* in 2 % peptone results in the formation of acids and ammonia, the amounts of both of which increase as the initial reaction of the medium is varied in the direction of increased alkalinity. Near the alkaline limiting value the production of acids is greater than that of ammonia.

10. The formation of indole is retarded by the presence of free alkali or acid in the medium.

11. The presence of certain sugars causes inactivity of the proteolytic enzyme produced by the bacillus and thus inhibits the formation of indole.

12. Different carbohydrates exhibit different degrees of indole-inhibiting power. The addition of 2 % glucose to peptone media produces complete inhibition of indole formation; 2 % lactose or 2 % maltose produce almost complete inhibition, while that produced by 2 % saccharose or 2 % mannite is only partial. 2 % starch possesses no inhibitory power.

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III. OBSERVATIONS ON THE ALBUMINOID AMMONIA TEST.

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SOME time ago in this laboratory it was found that effluents of fair and even very good quality were yielding albuminoid ammonia figures of 1.0 to 2.0 parts per 100,000, while pure drinking water gave figures varying from 0.02 to 0.1.

The albuminoid ammonia figures of good effluents generally approximate to 0.15, while in the case of pure waters the figures are generally less than 0.005.

It was evident that the technique involved some very considerable error, which quite vitiated the value of the test, and it was necessary to make an investigation to ascertain the cause of these abnormally high figures.

The distilling apparatus seemed to be quite above suspicion, as the corks were securely covered with tin-foil and there was no difficulty in obtaining ammonia-free water. Furthermore, the free and saline ammonia figures were normal.

Some impurity in the chemicals used in the albuminoid ammonia determination was therefore suspected. It had been observed that the permanganate-alkali mixture was very difficult to free from ammonia, but after two days' boiling it was always apparently possible to free the mixture by a single distillation in the apparatus.

The great difficulty experienced suggested however that possibly the mixture was not after all free from nitrogenous material and that by diluting and redistilling with ammonia-free water further yields of ammonia might be obtained.

It was resolved to test this possibility by experiment. Potassium permanganate and caustic soda in the usual proportion of $\frac{1}{2}$ g. to 10 g. respectively were boiled together in a small flask with ammonia-free distilled water for two days. The mixture was then poured into a litre of ammonia-free water in the distilling apparatus and distillation was carried out. Large amounts of ammonia were generated, e.g. 0.0001 g. When the ammonia ceased to come over, the ammonia-free distillate was collected and the contents of the flask were concentrated to a small bulk. The ammonia-free distillate was next

poured back and the distillation recommenced. As much ammonia was again produced as in the first distillation. This could often be repeated for several days; sometimes however the generation of ammonia ceased after a few distillations. Taking 0.0001 g. as a typical yield from *one* distillation, as 500 cc. of a drinking water are used in the albuminoid ammonia determination, this would correspond to the enormous error of 0.02 parts per 100,000. Occasionally a single distillation yielded even larger amounts, and the error was correspondingly increased.

It was evident that this was at any rate one source of error. The permanganate-alkali mixture could apparently be cleared of ammonia by a single distillation, but when added to the water being analysed the dilution and boiling would lead to the production of more ammonia, sufficient in amount to give altogether inaccurate results.

Prolonged boiling rarely led to the purification of the mixture, and it was concluded that a stable nitrogenous impurity was present which only decomposed with extreme slowness. The condition favourable to decomposition was great dilution, while concentration stopped the process entirely.

Through the kindness of Prof. Harden, a sample of permanganate-alkali mixture was obtained from a laboratory in which *normal* albuminoid figures were being obtained. When 50 cc. of this solution (containing $\frac{1}{2}$ g. of permanganate and 10 g. of alkali) was distilled with ammonia-free water, only 0.000005 g. ammonia was generated in each distillation. This corresponded to the small error in the albuminoid-ammonia figure of a water of 0.001 parts per 100,000.

It was thought that this mixture could be employed to ascertain whether the impurity existed in the permanganate or the soda in the contaminated mixture which was yielding the high results.

Accordingly 50 cc. of the pure mixture was boiled with $\frac{1}{2}$ g. of the suspected permanganate for two days and the solution was then distilled with ammonia-free water in the ammonia apparatus. Large amounts of ammonia were generated, *e.g.* 0.0001 g. ammonia in each distillation. This pointed to the existence of a nitrogenous impurity in the permanganate.

The pure mixture was next separately heated with 10 g. of the suspected soda and distilled as before. During each distillation only small amounts of ammonia were produced, *e.g.* 0.000005 g.

The impurity was thus chiefly concentrated in the permanganate.

Experiments were next carried out with the same brand of permanganate and soda as constituted the *pure* mixture. A solution was prepared in this laboratory, and after one day's boiling it generally yielded an error of about 0.001 to 0.002 parts per 100,000 in an albuminoid ammonia determination for a water. On one occasion the error was 0.003.

Some control experiments were also carried out to ascertain the amount of albuminoid ammonia generated from strychnine when decomposed by the permanganate-alkali. The alkaloid yields half its nitrogen content as albu-

minoid ammonia. The results as would be expected were about 10 % high, since if 0.0001 g. were produced from the strychnine this would be increased to about 0.00011 owing to the trace of nitrogenous impurity in the permanganate.

For ordinary work this would be sufficiently accurate, but for very important work it would be advisable to make a control estimation, under the same conditions of dilution and rate of boiling, of the ammonia yielded in one distillation of the permanganate-alkali mixture with ammonia-free water. This figure could then be subtracted from the figure obtained for the water or sewage effluent which was being analysed.

When fresh supplies of chemicals come into the laboratory, tests should be made before using them in routine work to ascertain whether the organic impurity is present in unusually large amount.

The presence of this oxidisable impurity may be one explanation of the observed decomposition of about 5 % of the permanganate in the control distilled water test carried out in the determination of the Tidy figure or oxygen absorbed in 4 hours at 27° C.: *e.g.*

Initial titration 10 cc. *N*/80 permanganate, equals 14.9 cc. standard thiosulphate.

(10 cc. permanganate,
10 cc. 25 % H_2SO_4 ,
100 cc. distilled water.)

Titration after 4 hours' incubation at 27° C. equals 14.2 cc. thiosulphate.

SUMMARY.

1. Potassium permanganate may contain a stable nitrogenous impurity which cannot as a rule be removed by prolonged boiling with alkali.

2. The impurity is not decomposed at all in concentrated alkaline solution, but gradually decomposes when the solution is considerably diluted. Consequently permanganate-alkali mixture can apparently be freed from ammonia by boiling with water, but when the resulting concentrated solution is again diluted and distilled, ammonia may once more be liberated in large quantities. When the mixture apparently freed from ammonia is boiled with the water, the albuminoid ammonia figure of which is being determined, the yield of ammonia is thus greatly augmented by that liberated from the permanganate.

3. The error involved may be so great as to vitiate the value of the albuminoid ammonia test altogether.

4. In the case of a purer brand of permanganate the error is reduced to about 0.002 parts albuminoid ammonia per 100,000.

5. It is essential in routine work to test fresh supplies of chemicals to ensure that the impurity is not present in excessive amount and in very accurate work a control experiment should be made during each determination of albuminoid ammonia.

IV. THE COMPOSITION OF STARCH. PART I. PRECIPITATION BY COLLOIDAL IRON. PART II. PRECIPITATION BY IODINE AND ELECTROLYTES.

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THE experiments recorded in this paper deal with the effects produced by (a) colloidal iron and (b) iodine on a solution of potato starch in water. The starch solution was obtained by adding starch suspended in a small volume of cold water to the required volume of boiling water and continuing the boiling for one minute.

I. THE PRECIPITATION OF STARCH BY COLLOIDAL IRON.

There are considerable differences of opinion regarding the nature of starch granules, but, generally speaking, starch is now regarded as consisting of two substances. Nägeli who investigated the action of hydrochloric acid and amylolytic enzymes on starch proposed the names amylogranulose and amylocellulose to designate them. Amylogranulose, the chief constituent of starch, goes into solution under the action of hydrolytic agents and gives a blue colour with iodine. Amylocellulose on the other hand does not go into solution under these conditions and is not coloured blue by iodine. This work has been extended by Maquenne and Roux [1903, 1905] and Fernbach and Wolff [1904]. From their experimental results they deduce that starch granules consist of two substances, (a) amylose and (b) amylopecten. Amylose, the chief constituent of starch, is insoluble in cold water but completely soluble in water boiled under pressure. On cooling such a solution the soluble amylose tends to revert to the insoluble form. Amylopecten, on the other hand, is of an entirely different nature. It swells up without dissolving when heated with water and is not coloured blue with iodine.

The results recorded in the following pages indicate that the soluble portion of starch (amylogranulose) may be further differentiated into a series of fractions.

(i) *Precipitation by colloidal iron only.*

The investigations of Bottazzi and Victoroff [1910] on the electrical state of starch in solution indicate that starch is electrically neutral. This conclusion is not supported by the effects observed when colloidal iron is added

to a solution of starch. Under these conditions three well marked phases may be recognised: (i) a portion of the starch is precipitated by the colloidal iron only, (ii) a second portion of the starch is carried down with the colloidal iron when an electrolyte is added to the solution, and (iii) the filtrate from (ii) contains starch which is not precipitated by colloidal iron either in the presence or absence of electrolytes.

The amount of starch precipitated by colloidal iron only is shown in the following experiment.

Colloidal iron was added to 1 % starch in the following proportions:

Colloidal iron	Starch 1 %	H ₂ O
4 cc.	20 cc.	16 cc.
8 cc.	20 cc.	12 cc.

A gelatinous precipitate was produced in each case. This precipitate was allowed to settle, after which the amount of starch remaining in the clear fluid, and the amount of starch in the original solution, were determined. In both cases it was found that 80 % of the original starch was precipitated by the colloidal iron. The amount of starch precipitated was independent of the amount of iron added. The lower limit, giving the minimal amount of iron necessary for precipitation, was not determined. Precipitation presumably depends upon the neutralisation of the negative charge on the starch by the positive charge on the colloidal iron. It became of interest therefore to determine how far the precipitation limits of colloidal iron by electrolytes were influenced by the addition of a solution of starch to it.

The minimal quantity of potassium sulphate required to precipitate 1 cc. of colloidal iron is given in the following figures.

Colloidal iron	H ₂ O	M/10 K ₂ SO ₄	
1 cc.	8.95 cc.	0.05 cc.	No precipitation
	8.9	0.1	"
	8.8	0.2	"
	8.6	0.4	"
	8.5	0.5	Complete precipitation

With 0.4 cc. M/10 K₂SO₄ there was no formation of distinct granules, but there was an obvious change in the appearance of the solution viewed by partially reflected light.

The same experiment was repeated except that 5 cc. of starch replaced 5 cc. of added water, thus:

Colloidal iron	Starch 1 %	H ₂ O	M/10 K ₂ SO ₄	
1 cc.	5 cc.	4 cc.	0.0 cc.	No precipitation
		3.95	0.05	"
		3.9	0.1	"
		3.8	0.2	"
		3.6	0.4	Partial precipitation
		3.5	0.5	Complete "

In the first four tubes there was a precipitate of starch, but this precipitate was greatly augmented by precipitated iron in the fifth tube. In the sixth tube

complete precipitation of the iron had been produced. The partial precipitation of the colloidal iron in the presence of starch by 0.4 cc. K_2SO_4 ($M/10$) compared with the amount 0.5 cc. K_2SO_4 ($M/10$) required when no starch was present indicates that the starch had diminished to a small degree the quantity of electrolyte required for precipitation. The results show that the electric charge on starch in solution is negative, though small in comparison with the electropositive charge on colloidal iron.

(ii) *The precipitation of starch by colloidal iron in the presence of electrolytes.*

The amount of starch precipitated from solution by colloidal iron is increased by the presence of electrolytes. But however much colloidal iron may be used, and however much precipitating electrolyte may be added, some starch remains in the filtrate. The amount of starch *not* precipitated by colloidal iron in the presence of electrolytes was determined.

To 100 cc. of approximately 1 % starch, 20 cc. of colloidal iron were added. Five minutes later 1.2 cc. of K_2SO_4 (M) were added as the precipitating electrolyte. The resultant filtrate was water clear in appearance but gave a deep blue colour on the addition of iodine. The quantities of starch in the original solution and filtrate were determined by hydrolysis to dextrose. The results showed that 89 % of starch was precipitated whilst 11 % remained in solution. Now a previous experiment had shown that on the addition of colloidal iron only to the starch solution 80 % of the starch was precipitated. We must therefore conclude that 9 % of the original starch is carried down by colloidal iron precipitated by electrolytes. The results show that three types of starch must be recognised as present in a solution of potato starch in water: (α) starch precipitated from solution by colloidal iron only; (β) starch taken down with colloidal iron when precipitated by a divalent negative ion; (γ) starch not affected by colloidal iron in any way. The results given above show that a solution of potato starch contains 80 % of (α), 9 % of (β), and 11 % of (γ). It may be remarked that the first fraction (α) includes the insoluble constituent of starch (amylocellulose) since this insoluble constituent would be mechanically carried down by the soluble starch precipitated by the colloidal iron. On this assumption the starch granule consists of amylocellulose and amylogranulose (α), (β) and (γ), the terms (α), (β) and (γ) designating those varieties which are precipitated by (α) colloidal iron only, (β) colloidal iron in the presence of electrolytes, and (γ) not precipitated by colloidal iron.

This, however, does not exhaust the possibilities of the substances contained in the starch granule, since precipitation by iodine in the presence of electrolytes, the amount of iodine added being just short of that required for complete precipitation of the starch, gives a filtrate which is coloured brown by iodine. This colour is due to the presence of dextrin in the starch solution. The presence of dextrin might be due to its production when the starch is dissolved in boiling water, but against this hypothesis is the fact that glycogen dissolved in cold water gives similar results. The complete precipitation of

starch by equivalent quantities of iodine in the presence of electrolytes shows that the granule contains no *soluble* substances which do not react with iodine. It appears reasonable to conclude that the starch granule contains a large number of polymers of the general formula $(C_6H_{10}O_5)_n$ increasing in complexity from dextrin to cellulose, the dextrin and cellulose being present in small quantities whilst amylogranulose (α), (β) and (γ) form the bulk of the granule. Probably other methods of precipitation would differentiate the amylogranulose (α) into a series of fractions.

II. THE PRECIPITATION OF STARCH BY IODINE AND ELECTROLYTES.

When iodine dissolved in potassium iodide is added to a solution of starch in water two characteristic changes may be observed: (i) the solution assumes a deep blue colour, and (ii) the lyophilic emulsoid colloid of starch is changed into a suspensoid colloid of marked lyophobic character.

A considerable amount of experimental work has been done to determine the nature of the changes involved. Owing to the varying content of "starch iodide" the chemical aspect of the problem has given place to views in which the physical element predominates. Küster [1894] put forward the hypothesis that iodide of starch consists of a solid solution of iodine in starch, and explained in this way the varying content of the iodine in starch iodide with the iodine concentration of the solution with which it was in equilibrium. Harrison [1911] on the other hand considered that in the case of iodine and starch an adsorption compound was formed. The most recent work on this subject has been done by Barger and Field [1912] in their analyses of the phenomena observed in the formation of blue adsorption compounds of iodine with various substances. From their experimental work they conclude that on the addition of iodine to starch there is a considerable adsorption of iodine, that the presence of electrolytes (potassium iodide) is necessary to bring about this adsorption, and that the blue substances formed behave as negative suspension colloids which have been rendered lyophobic by the iodine adsorbed.

The electronegative nature of starch iodide was shown by the experiments of Padoa and Savaré [1906] in which starch iodide moved to the anode when placed in an electric field. The experiments of Barger and Field on the precipitation of starch iodide by cations of inorganic salts confirmed this deduction as to the electronegative nature of starch iodide and demonstrated that the addition of iodine to starch changed a lyophilic emulsoid colloid (the starch solution) to a lyophobic suspensoid colloid (starch iodide). The facts that starch dissolved in water forms an emulsoid colloid and carries a small negative charge, that the reacting iodine dissolved in potassium iodide is presumably ionised and negatively charged, and that the resulting starch iodide forms a suspensoid colloid and is also negatively charged, militate against the acceptance of a purely physical hypothesis for the explanation of those changes which occur when iodine is added to starch. The experiments recorded in the

following pages on the effects observed when iodine dissolved in potassium iodide is added to a solution of starch indicate that the iodine first reacts quantitatively with the starch producing a definite chemical compound and that the starch iodide thus formed is a lyophobic suspensoid colloid which adsorbs iodine from solution according to the recognised laws of adsorption.

(A) *The quantitative relation of iodine to starch.*

The following experiments indicate that on the addition of small quantities of iodine to starch a reaction takes place in accordance with the recognised laws of chemical action.

(i) *Varying iodine.* Varying quantities of iodine were added to 10 cc. of a 1 % solution of starch contained in a series of tubes, and the resulting starch iodide was precipitated from solution by a small quantity of magnesium sulphate. After an interval of five minutes the contents of the tubes were filtered and the filtrates were tested for excess of iodine or starch. The following figures show the results obtained in a typical experiment:

Starch 1 %	Iodine 1 %	H ₂ O	MgSO ₄ (M)	Filtrate
10 cc.	0.3 cc.	9.6 cc.	0.1 cc.	Excess of starch
10	0.4	9.5	0.1	" "
10	0.5	9.4	0.1	" "
10	0.6	9.3	0.1	" "
10	0.7	9.2	0.1	Nil
10	0.8	9.1	0.1	Excess of iodine
10	0.9	9.0	0.1	" "

An experiment done under these conditions shows that when iodine is added to starch, and the lyophobic starch iodide is precipitated from solution by a divalent ion, a point is reached at which the filtrate contains neither starch nor iodine. Previous to this point the filtrate contains an excess of starch, and after this point the filtrate contains an excess of iodine. It is difficult to postulate an hypothesis which would explain these results on the basis of physical adsorption. The occurrence of a definite point at which the filtrate contains neither starch nor iodine suggests that iodine reacts chemically with the starch, and that at this point there is a definite chemical equivalence of the starch and iodine. This deduction is strengthened by the experimental observations that the equivalent point is not affected by dilution, temperature, or precipitating electrolyte.

(ii) *Varying concentration of starch.* In the following experiment the quantity of starch, contained in a final volume of 20 cc., was varied from 10 cc. of a 1 % solution to 10 cc. of a 0.2 % solution. The same quantity of precipitating electrolyte (MgSO₄) was added in each case and the quantity of iodine required to produce a filtrate containing neither starch nor iodine determined:

1 % starch	H ₂ O	I 0.1 %	MgSO ₄ (M)	Filtrate
10 cc.	5.5 cc.	4.75 cc.	0.2 cc.	Excess iodine
10	5.75	4.5	0.2	No iodine

0.8 % starch	H ₂ O	0.1 % I	MgSO ₄ (M)	Filtrate
10 cc.	6.2 cc.	3.8 cc.	0.2 cc.	Excess iodine
10	6.4	3.6	0.2	No iodine
0.6 % starch	H ₂ O	1.0.1 %	MgSO ₄ (M)	Filtrate
10 cc.	7.2 cc.	2.8 cc.	0.2 cc.	Excess iodine
10	7.4	2.6	0.2	No iodine
0.4 % starch	H ₂ O	1.0.1 %	MgSO ₄ (M)	Filtrate
10 cc.	8.2 cc.	1.8 cc.	0.2 cc.	Excess iodine
10	8.4	1.6	0.2	No iodine
0.2 % starch	H ₂ O	1.0.1 %	MgSO ₄ (M)	Filtrate
10 cc.	9.0 cc.	1.0 cc.	0.2 cc.	Excess iodine
10	9.2	0.8	0.2	No iodine

The mean values obtained from the above results are as follows:

% starch	Cc. of iodine required	Iodine/starch
10 cc. of 1 %	4.625	4.6
10 cc. of 0.8 %	3.7	4.6
10 cc. of 0.6	2.7	4.5
10 cc. of 0.4	1.7	4.25
10 cc. of 0.2	0.9	4.5

The direct relation between the amount of starch and the quantity of iodine required to precipitate it in the presence of an electrolyte again indicates the chemical equivalence of the starch and iodine.

Similar experiments were made in which the precipitating electrolyte was varied from 0.2 cc. MgSO₄ (M/10) to 0.2 cc. MgSO₄ (M). There was no change in the point at which the filtrate contained neither starch nor iodine. Similarly varying the temperature at which the experiment was done from 15° to 40° did not influence the quantitative values of the reaction.

(iii) *The iodine equivalent of starch.* All these results indicate that when iodine is added to starch a quantitative reaction takes place between the starch and the ionised iodine. The iodine equivalent of starch was determined from an experiment in which the amounts of starch and iodine in the solutions used had been accurately determined.

It was found that when 10 cc. of a starch solution containing 0.087 g. of starch were treated with 0.0068 g. of iodine the filtrate obtained after precipitation by MgSO₄ contained neither starch nor iodine. Therefore, on the above hypothesis of the chemical interaction of starch and iodine,

0.0068 g. of I is equivalent to 0.087 g. of starch,
or 127 g. of I is equivalent to 1635 g. of starch.

Now the sum of the atomic weights of C₆H₁₀O₅ is 162; and the empirical formula for starch has been assumed to be (C₆H₁₀O₅)_n. Therefore the least value of n, assuming that one molecule of starch reacts with one atom of iodine, is 10.

The experimental results obtained on adding colloidal iron to starch show that starch contains a variety of polymers. The value n = 10 can therefore

be taken only as a mean value for a number of starch complexes in which n is continually varying.

(iv) *The iodine equivalent of starch granulose (γ)*. The iodine equivalent of starch granulose (γ) was determined in the way described in the previous experiment. The following results give the details of an experiment:

100 cc. of starch were precipitated by 20 cc. of colloidal iron and 1.2 cc. K_2SO_4 (M). 5 cc. of the resulting filtrate containing starch granulose (γ) were put into a series of tubes to which were added varying quantities of iodine and $MgSO_4$ ($M/10$) to make the volume 10 cc. The filtrates were tested with starch and iodine.

Starch granulose (γ)	H_2O	0.1 % I	$MgSO_4$ ($M/10$)	Filtrate
5 cc.	1.9 cc.	3.0 cc.	0.1 cc.	Excess of iodine
5	2.9	2.0	0.1	“ ”
5	3.9	1.0	0.1	“ ”
5	4.1	0.8	0.1	Nil
5	4.3	0.6	0.1	Excess of starch

The original starch solution gave as equivalent quantities 5 cc. starch and 4 cc. iodine. The amount of starch granulose (γ) contained in the filtrate was 10.6 % of the total starch contained in the original solution.

Therefore the iodine equivalent of starch granulose (γ) compared with that of an equal quantity of the original starch was approximately as two to one. In other words if 1600 g. of the original starch were equivalent to a gram atom of iodine, approximately 800 g. of starch granulose (γ) were equivalent to the same quantity of iodine.

In the case of starch $(C_6H_{10}O_5)_{10}$ is equivalent to I and for starch granulose (γ) $(C_6H_{10}O_5)_5$ is equivalent to I.

(B) *The adsorption of iodine by starch iodide.*

In the foregoing experiments the amount of iodine added to the starch solution before the addition of the precipitating electrolyte was relatively small, and under these circumstances it has been shown that the reaction passes through a definite stage previous to which there is an excess of starch, and after which there is an excess of iodine, in the solution. This result is not compatible with the hypothesis that starch adsorbs iodine *ab initio*. The question however arises whether starch iodide when formed further reacts with an excess of iodine, contained in the solution, in a chemical or physical way. Substances containing a variable quantity of iodine in relation to the starch have been isolated and this fact suggests that a physical process enters into the reaction after the formation of the starch iodide compound. The following experiment illustrates the relation of the iodine contained in the filtrate and that added to the original starch solution when the quantity of added iodine is continuously varied.

Starch 1 %	Iodine 1 %	H ₂ O	MgSO ₄ (M)	Excess of I in filtrate	Iodine precipitated
25 cc.	1.5 cc.	22.5 cc.	1 cc.	(starch in filtrate)	1.5 cc.
25	2.0	22.0	1	0	2.0 (x)
25	2.5	21.5	1	0.15 cc.	2.37
25	5.0	19.0	1	0.89	4.11
25	7.5	16.5	1	2.8	4.7
25	10.0	14.0	1	5.03	4.97
25	12.5	11.5	1	6.9	5.6

The figures show that after the equivalent point (x) at which the filtrate contains neither starch nor iodine, the starch iodide precipitated contains a varying quantity of iodine, the amount of iodine in the "starch iodide" continually increasing as the iodine content of the original mixture increases. In fact the figures show that after the point (x) the precipitated "starch iodide" takes down iodine with it from solution according to the recognised laws of adsorption. Therefore in the action of iodine on starch two separate and distinct phases may be recognised.

(1) The iodine reacts with the starch in a quantitative manner, approximately 1 gram-atom of iodine being equivalent to 1600 g. of starch or 800 g. of starch granulose (γ).

(2) The lyophobic starch iodide thus formed when precipitated by electrolytes adsorbs iodine from solution according to the recognised laws of adsorption provided an amount of iodine is present in excess of that required for the first reaction.

SUMMARY.

1. Precipitation of starch by colloidal iron shows that starch granulose can be separated into three fractions, (α), (β) and (γ), forming 80 %, 9 % and 11 % respectively of the starch granulose. (α) is precipitated by colloidal iron only, (β) by colloidal iron and electrolytes, and (γ) is not precipitated by colloidal iron under any conditions.

2. Precipitation of starch by iodine and electrolytes shows (a) that starch contains an insoluble constituent which does not react with iodine (amylocellulose), (b) that all the soluble constituents of starch are precipitated by iodine in the presence of electrolytes, and (c) that the final fraction precipitated by iodine gives a brown colour with iodine.

3. The results (1) and (2) indicate that starch contains a variety of polymers varying in complexity from amylopectin to amylocellulose, the relative quantities of dextrin and cellulose being small whilst the bulk of the granule is composed of amylogranulose (α).

4. Iodine reacts with starch in a quantitative manner forming starch iodide. Approximately 1600 g. of starch are equivalent to 127 g. of iodine, or $(C_6H_{10}O_5)_{10}$ is equivalent to a gram-atom of iodine. In the case of starch granulose (γ) approximately 800 g. react with 127 g. of iodine; or $(C_6H_{10}O_5)_5$ is equivalent to a gram-atom of iodine.

5. Starch iodide adsorbs iodine from solution so that after the equivalent point the amount of iodine contained in the precipitated starch iodide is a function of the amount of iodine contained in the original solution.

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V. OBSERVATIONS ON THE ACCURACY OF DIFFERENT METHODS OF MEASURING SMALL VOLUMES OF FLUID.

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(Received February 13th, 1919.)

EVERY serologist has his own favourite method of measuring the small volumes of different fluids which he needs in performing complement fixation reactions or in making a series of serum dilutions for agglutination. He usually regards the method to which he is accustomed as the best and may resent any imputation on its accuracy, but few serologists take the trouble to check the method they employ by careful control observations. There is no convenient method for determining the percentage of serum in a dilution sufficiently closely, but there are plenty of chemical substances which can be substituted for serum. One cannot, indeed, carry out volumetric analyses on the small volumes involved in Wright's method of preparing serum dilutions in a capillary pipette, but one can use larger volumes in the same way and analyse the results satisfactorily and can very safely reason that errors detected with the larger volumes are likely to be exaggerated with the smaller.

I have lately had occasion to enquire into possible errors in my own serological technique and I have carried out a certain number of observations, as carefully as I was able, on the lines suggested above. Although there is nothing astonishing in the results it may be of service to put them on record. It has been a gain to me to ascertain the degree of my own error and it would probably be a useful exercise for any serologist to check his methods in similar fashion.

The fluids chosen for measurement were (1) a saturated solution of iodine in saturated potassium iodide solution, and (2) a not quite saturated solution of calcium chloride in water. The latter was on the whole the more satisfactory, being cleaner to work with. The dilutions were titrated in the ordinary way, the iodine with $N/10$ and $N/100$ thiosulphate and a starch indicator, and the calcium chloride with $N/10$ and $N/100$ silver nitrate and a potassium chromate indicator. My thanks are due to Dr W. H. Hurtley, Lecturer on Chemistry at St Bartholomew's Hospital, for kind advice and help in the matter.

There are two ways of measuring fluids, apart from weighing, which is out of the question for serological work. One is measurement by pipette, and the

other measurement by drops, and I was anxious to compare the accuracy of these two methods.

In measuring by pipette the serologist is at a disadvantage as compared with the chemist. Volumetric analysis has been developed into a fine art by generations of chemists and the degree of accuracy obtainable is very high. The chemist has his standard burettes and pipettes and has been trained how to use them. Unless the serologist has been trained as a chemist he is apt to be less scrupulous in the use of the instruments at his command, and he lies under the further disadvantage that he commonly has to measure very small quantities of fluid by means of instruments much smaller and more liable to error than those used by the chemist. The first problem that arises is therefore the degree of error likely to be present in careful pipette measurements of small volumes of fluid, ranging from 1 cc. to 0.01 cc., such as the serologist often has to employ.

THE RANGE OF ERROR IN SMALL PIPETTE MEASUREMENTS.

Those who work with graduated 1 cc. and 0.1 cc. pipettes usually employ as motive force either a rubber teat or a mouthpiece connected with the pipette by a rubber tube. Some such method is necessary with a 0.1 cc. instrument, though with a 1 cc. pipette one can work by gravity, controlling the flow with the finger only.

To check the accuracy of delivery of small volumes from such pipettes the following procedure was adopted. A concentrated solution of calcium chloride was carefully titrated against $N/10$ AgNO_3 : 1 cc. of the CaCl_2 solution was found equivalent to 61.4 cc. $N/10$ AgNO_3 ; the measurement of the 1 cc. was performed with great care by Dr Hurtley. Two Lautenschläger pipettes were washed out with strong sulphuric acid and then repeatedly with distilled water: one was a 1 cc. pipette graduated in tenths and hundredths, the other a 0.1 cc. pipette graduated in hundredths and two-hundredths. A well-fitting rubber teat was attached to each. I then attempted to deliver into evaporating dishes amounts of the calcium chloride solution varying from 1 cc. to 0.01 cc., and titrated them against AgNO_3 , using a decinormal solution for the larger and a centinormal one for the smaller amounts. Even 0.01 cc. of the calcium chloride solution required 6 cc. of centinormal AgNO_3 , so that the titration could be carried out with considerable accuracy. At the same time my colleague, Dr R. G. Canti, who was provided with a similar pair of Lautenschläger pipettes, which he was accustomed to use with a mouthpiece and rubber tube, performed similar measurements. There was thus an opportunity for comparing the teat with the mouthpiece as a motive force.

The results of this experiment are shown in the following table. In the second column is shown the expected value of each amount, assuming the correctness of the preliminary titration, in the third and fifth columns the value actually found, and in the fourth and sixth the percentage by which the value found differed from what it ought to have been.

Table I. *Errors in measurements with small graduated pipettes.*

Measurements with 1.0 cc. pipette.					
Amount measured	Correct value	F. W. A. using teat		R. G. C. using mouthpiece	
	in cc. $N/10$ $AgNO_3$	Value found	% deviation	Value found	% deviation
1.0 cc.	61.4	60.8	- 0.98	60.0	- 2.28
1.0	61.4	61.9	+ 0.81	62.0	+ 0.97
1.0	61.4	61.1	- 0.49	—	—
0.5	30.7	30.5	- 0.66	—	—
0.1	6.14	5.7	- 7.17	6.0	- 2.28
0.1	6.14	5.75	- 6.36	—	—
Measurements with 0.1 cc. pipette.					
0.1 cc.	6.14	6.0	- 2.28	6.15	+ 0.16
0.1	6.14	—	—	6.5	+ 5.86
0.05	3.07	2.95	- 3.91	3.015	- 1.79
0.01	0.614	0.605	- 1.47	0.68	+ 10.74
0.01	0.614	0.75	+ 22.15	0.775	+ 26.22
0.01	0.614	0.635	+ 3.42	0.74	+ 20.52

The chief interest of these figures is that they represent the actual results obtained by two ordinary workers, reasonably practised in pipette measurements, and done with extra care because there was a certain emulation as to who could produce the best results. Doubtless there are many workers who could do better: we only regard ourselves as representing the average¹.

It is evidently possible to measure 1 cc. or 0.5 cc. with reasonable accuracy: my own four measurements of this order were all within 1 % of the correct amount. Attempts to deliver 0.1 cc. from a 1 cc. pipette were less successful. This quantity can be more accurately delivered from a 0.1 cc. instrument, and one of Dr Canti's efforts was almost exact; but he failed to repeat it. The margin of error is clearly greater with the smaller instrument, and when it comes to delivering 0.01 cc. the error is apt to become a serious one. The grosser errors were always in the direction of delivering too much, probably owing to transfer of fluid on the exterior of the nozzle. It is clearly unwise for the average worker to rely on the measurement of one hundredth of a cc. from a pipette with any accuracy.

Two further points came out in the course of this experiment, though they are not indicated in the table. A slightly larger volume is delivered when the pipette is held vertical and emptied slowly. In delivering one-tenth of the total graduated length, more accuracy is attained by measuring one of the middle tenths than the terminal one.

¹ I communicated my results to Dr Carl Browning of the Middlesex Hospital, who thereupon undertook some similar observations—using a narrow 0.1 cc. pipette, provided with a rubber tube and mouthpiece. The fluid used was hydrochloric acid, and the measured volumes were titrated against $N/300$ sodium hydrate. Twenty successive measurements of 0.01 cc. were made, and of these sixteen differed from the mean by less than 2 %, while the extreme variations from the mean were less than 5 %. Eighteen measurements of 0.03 cc. were also made, and fifteen of these were within 1.3 % of the mean, while the extreme variations from the mean were 2.9 %. These efforts are clearly better than those of Dr Canti and myself.

ERRORS INCIDENT TO THE PREPARATION OF A SERIES OF DOUBLING
DILUTIONS.

Inasmuch as this is one of the commonest procedures in agglutination work, considerable interest attaches to the accuracy of the different methods by which it can be carried out, and especially to a comparison of the pipette and drop methods. I therefore performed a number of experiments with this object in view.

In order to have an idea of the standard of accuracy which might be attainable I invited Dr Hurtley to prepare me a progressive series of doubling dilutions of the same calcium chloride solution as was used in other experiments. The solution was at this time slightly stronger: (this was the first time it had been used); the preliminary titration showed that 1 cc. = 61.6 N/10 AgNO₃. Dr Hurtley prepared the dilutions with great care, employing all the precautions incident to volumetric analysis, and even washing out and drying the pipette between successive measurements. The volume measured at each step was 2.5 cc. Five dilutions were prepared, from 1 in 2 to 1 in 32, each being made from the one preceding it, so that any error might be cumulative. The results are shown in Table II.

Table II. *Deviations from accuracy in a series of dilutions prepared by a chemist.*

Primary solution; 1 cc. = 61.6 N/10 AgNO ₃ .			
Dilution	Correct figure	Result on titration	% deviation from correct figure
1 in 2	30.8	30.65	-0.5
1 in 4	15.4	15.45	+0.3
1 in 8	7.7	7.85	+1.9
1 in 16	3.85	3.80	-1.3
1 in 32	1.92	2.00	+4.1

The results seen in this table show that a trained chemist, using adequate methods, can prepare a series of desired dilutions with a high degree of accuracy. The slight apparent errors are now +, now -, which suggests that they may depend upon errors in titration rather than in the preparation of the dilutions.

But no serologist can work in this laborious way. He usually employs his pipette in quite a different manner. It is usually not a "delivery" pipette, but one graduated to the point and he blows out the contents as completely as he can. This process inevitably leads to error unless the instrument is dried out at each operation, and what is more, in preparing a series of doubling dilutions in the ordinary way, the error is cumulative. In order to get some idea of how great it may become, I made the two following experiments, using a delivery pipette in the manner of a serologist.

I took an ordinary 4 cc. pipette and measured into each of eight test tubes 4 cc. of distilled water, as carefully as possible but expelling the whole amount of fluid by blowing. The pipette was then dried out, and 4 cc. of strong calcium

chloride solution (1 cc. = 61.5 cc. $N/10$ AgNO_3) added to the first tube. The mixture was taken up into the pipette and expelled six times, to ensure thorough mixing and to wash out the pipette, and then, without any other washing or drying, 4 cc. of the mixture were transferred to tube 2 and the process repeated, and so on to the eighth tube, the pipette being at each stage washed out six times with the mixture just made. The results on titration were as follows:

Table III. *Showing cumulative error from incorrect use of pipette.*

Primary solution; 1 cc. = 61.5 cc. $N/10$ AgNO_3 .

Dilution	Correct figure	Result on titration	% deviation from correct figure
1 in 2	30.75	31.0	+ 0.81
1 in 4	15.375	15.825	+ 2.92
1 in 8	7.687	8.05	+ 4.72
1 in 16	3.843	4.0	+ 4.08
1 in 32	1.921	2.06	+ 7.23
1 in 64	0.960	1.04	+ 8.33
1 in 128	0.48	0.535	+ 11.45
1 in 256	0.24	0.275	+ 14.58

This series illustrates very well the progressive nature of the error in a short series of dilutions when the pipette is wrongly used. If it can amount to 14 % using so large a pipette as one of 4 cc., how much greater must it be with a capillary pipette in which the surface wetted bears a far larger proportion to the volume of fluid measured.

A method of preparing serum dilutions sometimes employed is as follows: Supposing one has three rows of tubes to fill, as in an ordinary T.A.B. agglutination, a tenfold dilution of the serum is prepared, and one volume placed in each of the first three tubes. Three volumes of the tenfold diluted serum are then put in a watch-glass and three volumes of saline added and mixed. A volume of this mixture is placed in each of the second three tubes, leaving three in the watch-glass, to which a further three volumes of saline are added for the third dilution and so on. The pipette is thus alternately wetted with pure saline and with the dilution at which one has arrived. If, on reaching the end, the volume remaining in the watch-glass is measured, it is never three volumes, but always less, by a half or a third of a volume.

I have tested the degree of inaccuracy of this obviously inaccurate method in the following manner. I used a home-made pipette of narrow glass tubing, holding about 2 cc. to the top mark: it was actuated by a rubber bulb. The solution employed was a strong one of iodine in potassium iodide. I put two pipettesful of water into a glass dish, washed out the pipette several times with iodine solution, and then added two pipettesful of iodine solution to the dish and mixed well. Two pipettesful were transferred to tube 1, and then two more of water added to the dish and mixed. Eight tubes were thus filled in succession. The results on titration with sodium thiosulphate are seen in Table IV.

Table IV. *Showing cumulative error from erroneous technique.*Primary solution; 1 cc. = 61.14 cc. *N*/10 thiosulphate.

Dilution	Correct figure	Result on titration	% deviation from correct figure
1 in 2	30.57	30.7	+ 0.42
1 in 4	15.28	15.1	- 1.8
1 in 8	7.64	7.45	- 3.8
1 in 16	3.82	3.49	- 8.7
1 in 32	1.91	1.63	- 14.7
1 in 64	0.955	0.795	- 16.8
1 in 128	0.477	0.36	- 24.6
1 in 256	0.238	0.175	- 26.5

Whereas in the preceding experiment the error was a progressive one on the + side, this one shows a similar and worse error on the - side. The direction of the error depends on the precise departure from the proper use of the pipette. These are not the only experiments of the sort which I have made, but all have shown the same sort of thing. These are selected and set out here in detail to illustrate the fact that an error which appears negligible in the individual measurement becomes a serious one when it is cumulative. I know of no way in which a serologist is likely to use a pipette which will avoid such error: this is not said in blame, for he has not the time to employ the technique of the trained chemist. But he ought to know what his error is liable to be.

THE DROP METHOD OF MEASURING.

All the sources of error which attend measurement by pipette are entirely avoided in measurement by drops, and in their stead we encounter the new set of difficulties which attends the delivery of equal drops. These however are more easily overcome than in the case of the pipette. Donald has shown us a simple technique by which, with a little practice, it is easy to deliver substantially equal drops of different fluids. [1915, 1916.]

The three important factors governing the size of a drop issuing from a vertically held pipette are (1) the external diameter of the nozzle, (2) the surface tension of the fluid, and (3) the rate of dropping. By the aid of a wire and drill gauge, such as can be bought at any good tool shop, pipettes of constant size can be made as they are needed. The variation in surface tension of different fluids, such as normal saline, fresh human serum, or phenolated rabbit serum, can be compensated by determining which holes in the gauge will furnish pipettes delivering sensibly equal drops of these fluids: once determined this has not to be done again. With a little practice any one can keep his drop rate reasonably constant at about one per second. All this can be read in detail in Donald's published papers.

There are two ways in which one may employ the method in measuring small volumes of fluids of different surface tension. With a single pipette one can determine the relative size of the drops furnished by each fluid, keeping the conditions constant—*e.g.* by ascertaining how many drops of each go to

make up 1 cc. It is then easy to calculate how many drops of each must be mixed to form any desired dilution. The objection to this way of working lies in the impossibility of measuring fractions of a drop. The second plan, that of calibrating two pipettes to deliver equal drops, is therefore much to be preferred.

In endeavouring to test the accuracy of the drop method, I prepared a series of eight doubling dilutions of strong calcium chloride solution. The drops of this solution and of distilled water were found to be so nearly equal in size (256 to 245) that I did not attempt to make two pipettes to deliver equal drops, but adopted the first of the two plans just mentioned; I used one pipette, calculating the numbers of drops required and neglecting fractions of a drop. I did not therefore employ the method in the best way; nevertheless, as will be seen from Table V, the results were surprisingly good.

Table V. *Showing smallness of error with drop method.*

Primary solution of CaCl_2 ; 1 cc. = 61.5 cc. $N/10$ AgNO_3 .

Dilution	Correct figure	Result on titration	% deviation from correct figure
1 in 2	30.75	31.25	+ 1.6
1 in 4	15.375	14.8	- 3.7
1 in 8	7.687	7.65	- 0.5
1 in 16	3.84	3.85	+ 0.3
1 in 32	1.92	1.95	+ 1.5
1 in 64	0.96	0.945	- 1.6
1 in 128	0.48	0.49	+ 2.1
1 in 256	0.24	0.265	+ 10.4

It will be seen that the errors here are, with the exception of the eighth dilution, of the same small order as in the series of dilutions prepared by Dr Hurltley by "chemical" use of the pipette (see Table II). The result is very much better than anything I myself could achieve with a pipette. On the first two occasions on which I tried to make this series of dilutions the results had been wide of the mark, owing to miscalculation of the relative size of the drops of the two fluids. After Mr Donald had been so kind as to visit my laboratory and to point out certain mistakes in technique, the thing became easy, and the above figures represent my third attempt. The dropping was done at constant rate from a burette, provided with a Marriott's tube to keep the pressure even, and not by hand.

The advantages claimed by Donald for his drop technique appear to me to be justified. Anyone can make and calibrate his own pipettes, easily and cheaply as they are required. There is no cumulative error in preparing an ascending series of dilutions. The drop method can be used with the same accuracy in measuring very small volumes as larger ones, whereas it is with the small measurements frequently required by the serologist that the pipette method shows up to such disadvantage. Lastly, provided that the conditions for its use are duly observed, the drop method seems intrinsically more accurate than measurement by pipette.

CONCLUSIONS.

The figures which have been given above are doubtless less exact than could have been obtained by weighing. Any errors which may have been incident to the process of volumetric analysis are of course superadded to those depending on faulty technique in the preparation of the series of dilutions. The errors of titration are probably, however, less than 1 % and I believe that the results obtained are of sufficient accuracy to allow of a fair estimate of the degrees of error which may attend the measurement of small volumes of fluid in serological work.

The chief conclusions which appear to follow from these few observations are as follows:

(1) Volumes of 1 cc. and 0.5 cc. can be measured by pipette with reasonable accuracy. The error attendant on the attempt to deliver 0.1 cc. may amount to 5 %, even when a 0.1 cc. pipette is employed. The delivery of 0.01 cc. from a pipette may be exceedingly inaccurate.

(2) The only way in which really accurate results can be obtained with a pipette in preparing an ascending series of dilutions, is to use it as a delivery pipette and to wash and dry it out between successive measurements. The alternative method, that of using it as a delivery pipette, mixing the dilution by shaking, and then washing out two or three times with the mixture, rejecting the washings, is one hardly adapted for serological work. Unless such precautions are taken the small error introduced, perhaps only one of 0.5 % in a single measurement, though probably much more with a capillary pipette, becomes magnified into one of 10 % or more at the eighth dilution.

(3) The drop method is greatly to be preferred, for serological work, to the pipette method, provided it is properly carried out with calibrated pipettes.

It may be urged that in a process such as the agglutination test, beset with liabilities to inaccuracy, an error of 10 % or so in the serum dilutions is not of much moment. For rough work perhaps it is not, but for accurate work it is. Improvement in agglutination technique can only be brought about by endeavouring to correct the individual sources of error which attend it. This is equally true of such methods as are involved in the technique of the Wassermann reaction. My excuse for publishing these few observations is that I have learned a good deal from them myself, and that it is possible that others may find them not without interest.

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VI. ON THE SEPARATION OF ANTITOXIN AND ITS ASSOCIATED PROTEINS FROM HEAT-DENATURATED SERA.

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(Received February 7th, 1919.)

It is recognised that in unheated antidiphtheritic and antitetanic sera the antitoxins are associated with the "salt-soluble" globulins precipitated between 30 and 50 % of saturation with ammonium sulphate. For this reason, in the routine concentration of such sera, it has been customary to isolate and dialyse the protein precipitated between these limits and, recently, observations have been published in regard to the factors limiting the degree of concentration thereby obtained [Homer, 1917, 1].

Further work has shown that, in heated sera, the association of the antitoxin with these so-called Second Fraction Precipitates is affected by the extent of the denaturation of the proteins induced during the preliminary heating of the serum. The heat-denaturation is evidenced by an increased precipitability of each of the individual serum proteins by ammonium sulphate, a phenomenon which is accompanied by an increase in the ratio of "salt-insoluble" to "salt-soluble" globulins and by an association of antitoxin with the "salt-insoluble" protein thus formed [Homer, 1917, 2, 1918, 1].

In the light of these observations it was necessary to extend the scope of the previous investigations so as to ascertain:

I. To what extent the limits for the precipitation with ammonium sulphate can be narrowed so as to include, in the Second Fraction Precipitates from heated sera, only those proteins with which the antitoxin is definitely associated.

II. Whether or no the antitoxin is evenly distributed throughout the protein fractions precipitated at successive stages between the limits indicated in (I).

I. THE LIMITS FOR THE PRECIPITATION OF THE SECOND FRACTION PRECIPITATES.

The gradient for the curves representing the precipitation of the serum proteins at progressively increasing percentages of saturation with ammonium sulphate indicates that there are no critical points for the precipitation of the eu- or of the pseudo-globulin or of the serum albumin.

The protein precipitated from unheated sera between 30 and 50 % of saturation with this salt consists mainly of pseudoglobulin admixed with small amounts of euglobulin and of albumin, whereas that similarly precipitated from heated sera contains a lower proportion of admixed euglobulin and a greater proportion of albumin. As was to be surmised, the relative proportion of the proteins precipitated from heated sera at this stage was influenced by the extent of the denaturation induced during the heating of the serum.

Experimental work was therefore undertaken to ascertain to what extent (a) the upper, and (b) the lower limit for the degree of saturation with ammonium sulphate could be changed so as to retain in the Second Fraction Precipitates only those proteins with which the antitoxin is definitely associated.

(a) The upper limit for the precipitation of the Second Fraction Precipitates.

The following observations show that the extent of the precipitation of albumin with the Second Fraction is a function of the heat-denaturation induced in the serum.

To separate volumes of plasma were added the requisite volumes of acid or of alkali to adjust the $[H]$ to suitable values. The separate sera, after being heated at a temperature of 57° for six hours, were made 50 % of saturation with ammonium sulphate and filtered. From the gravimetric determinations of the albumin content of the filtrates was calculated the percentage of albumin precipitated with the Second Fraction Precipitates. It was found that, where the heat-denaturation had been of the order of 60, 50, 45, 35 and 22 % respectively the Second Fractions contained 75, 55, 45, 40 and 22 % of the total albumin of the plasma.

As the antitoxin is attached to the pseudoglobulin and not to the albumin fraction of the serum proteins, the presence of albumin in the Second Fraction Precipitates must reduce the degree of concentration; its exclusion is, therefore, desirable.

It was found that the precipitation of heat-denaturated albumin could be eliminated by a sufficient lowering of the degree of saturation with ammonium sulphate. But, in the concentration of sera, where the main object is to ensure the complete recovery of the antitoxin in association with a minimum percentage of the proteins of the original plasma, such a procedure is not practicable. For, as there is no sharp line of demarcation between the precipitation of pseudoglobulin and of albumin from heat-denaturated sera, the lowering of the upper precipitation limit to the extent required for the complete exclusion of the albumin leads to considerable losses of antitoxin; the concentration of ammonium sulphate thereby adopted is insufficient to precipitate the whole of the pseudoglobulin and its associated antitoxin.

Thus, from sera in which a heat-denaturation of 35 % had been induced, there was a slight precipitation of denaturated albumin at 40 % of saturation

with ammonium sulphate; the extent of the precipitation rapidly increased with the further addition of ammonium sulphate. In order, therefore, to avoid the precipitation of albumin in the Second Fraction Precipitates it was necessary to lower the upper precipitation limit from 50 to 38 % of saturation.

On the other hand the precipitation of the pseudoglobulin and antitoxin from the same serum was not complete until the concentration of the sulphate had reached 44 % of saturation. Thus, the precipitate isolated between 30 and 42 % of saturation contained only about 90 % of the antitoxin, while rather less than 80 % of the antitoxin was associated with the protein precipitated between 30 and 40 % of saturation with the salt.

This difficulty was even more pronounced in the fractional precipitation of the antitoxin and its associated protein from sera showing a more extensive heat-denaturation. Thus, from sera showing a heat-denaturation of 50 % or more there was a marked precipitation of albumin even at 26 % of saturation with ammonium sulphate. It is obvious that in such cases the elimination of albumin from the end products is impracticable.

In dealing with the routine sera in which the heat-denaturation was of the order of 35 % or less, it was decided that the upper limit should not be reduced to less than 44 % of saturation. Even this reduction led to the exclusion of a considerable proportion of the denaturated albumin which would have been precipitated at 50 %, and, as a result, the end products in the former case showed a greater degree of concentration than was obtained in the latter. Thus, from a given serum, while the fraction isolated between 30 and 50 % of saturation with ammonium sulphate contained 33.3 % of the total serum proteins, that between 30 and 44 % of saturation contained only 25 % of the proteins. The load of antitoxin carried by the protein fraction thus isolated between the wider limits was of the order of 16,000 units and that between the narrower limits of 17,500 units per g. of protein.

(b) *The lower limit for the precipitation of the Second Fraction Precipitates.*

Having demonstrated that the degree of concentration is improved by the lowering of the upper limit for the precipitation of the Second Fraction by ammonium sulphate, the next step was to ascertain to what extent the lower limit could be raised so as to ensure the exclusion of that portion of the heat-denaturated globulin to which no antitoxin is attached.

The preliminary precipitation and separation of the First Fraction Precipitates (30 % of saturation with ammonium sulphate) from unheated sera remove most of the euglobulin together with a small amount of pseudoglobulin and antitoxin. The pseudoglobulin being "salt-soluble" can be recovered by the extraction of the precipitates with brine. On the other hand the corresponding precipitates from heated sera contain relatively greater amounts of heat-denaturated pseudoglobulin of which a certain proportion

has been converted into a "salt-insoluble" condition; the extent of the conversion is a function of the denaturation.

In sera showing a heat-denaturation of 40 % or less, the denaturated pseudoglobulin precipitated with the First Fraction Precipitates also carried down appreciable amounts of antitoxin but, as the latter was associated with salt-soluble protein, it could be recovered by an extraction of the precipitates with brine.

The precipitation of antitoxin from sera showing a more extensive heat-denaturation began at, and was completed at, a much lower concentration with ammonium sulphate than in the above. The proportion of antitoxin carried down with the First Fraction Precipitates increased progressively with the heat-denaturation. At the same time, owing to the increasing insolubility of the denaturated protein, there was a corresponding decrease in the percentage recovery of antitoxin and its associated proteins in a form suitable for clinical use. In fact, under some conditions, *e.g.* in sera more acid than P_H 4.5 or in sera containing excessive amounts of phenol and its homologues, or of ether, chloroform, etc., while practically the whole of the antitoxin is precipitated with the First Fraction Precipitates, only a small percentage of it can be recovered in solution, as the protein to which it is attached has become almost entirely "salt-insoluble."

From these observations it is clear that, in such sera, the lower limit for the precipitation of the Second Fraction with ammonium sulphate must be reduced rather than raised.

The attempts to increase the degree of concentration by raising the limit for the precipitation of the First Fraction Precipitates were, therefore, confined to such sera as showed a denaturation of less than 40 % and the problem was accordingly studied in respect of sera showing a denaturation (1) of from 25 to 40 %, and (2) of less than 25 %.

The end products from (1) were clear and readily filtrable. Those from (2) were cloudy and, owing to the suspension of heat-denaturated protein, could only be filtered with difficulty; they also showed a lower degree of concentration and a lower percentage removal of the serum proteins than was obtained from the sera in (1).

(1) *Sera in which a heat-denaturation of 25 to 40 % had been induced.*

It was found that in such sera the amount of protein precipitated with the Second Fractions isolated between 30 and 44 % of saturation with ammonium sulphate was not appreciably less than in those isolated between 33 and 44 % of saturation. On the other hand by raising the lower limit for the precipitation of the First Fraction Precipitates to 36 % of saturation, the amount of protein isolated between 36 and 44 % of saturation was only 70 % of that obtained between the wider limits. At the same time, however, there was a corresponding proportional increase (30 %) in the percentage of the antitoxin precipitated with the protein.

It is evident that no material gain resulted from the raising of the lower precipitation limit. On the contrary, the method of procedure was disadvantageous. It transpired that, while the antitoxin carried down with the First Fraction precipitated from the serum in question at 30 % of saturation with ammonium sulphate could be completely recovered by extraction of the precipitates with brine, that carried down with the precipitates at 33 and at 36 % of saturation with the sulphate could not be recovered to the same extent. In the latter cases it seems as though the greater concentration of *sulphions*, presumably by an electrical charging of the protein molecules, favours the conversion of the heat-denaturated pseudoglobulin and its associated antitoxin into a "salt-insoluble" condition: the antitoxin attached to the protein thus rendered "salt-insoluble" cannot be recovered in a form suitable for clinical use even though its activity has in no way been impaired.

(2) *Sera in which a heat-denaturation of less than 25 % had been induced.*

As was to be expected the raising of the lower limit for the isolation of the Second Fraction Precipitates from sera showing a heat-denaturation of 25 % or less led to a more marked diminution in the amount of protein precipitated than was shown in the sera discussed in (1).

Thus, in the cases investigated, the precipitate between 30 and 44 % of saturation with ammonium sulphate contained 15 and 48 % more protein than where the limits for precipitation had been 33-44 and 36-44 % of saturation respectively. Moreover, the products obtained from the dialysis of the protein precipitated between the widest limits were cloudy and unsatisfactory; those from the precipitates isolated between the narrower limits were clear, as the increased concentration of ammonium sulphate had given the particles of suspended heat-denaturated protein the necessary charge to ensure their aggregation, precipitation, and separation with the First Fraction Precipitates.

The determination of the antitoxin associated with the Second Fraction Precipitates thus isolated showed that while those precipitated between 30 and 44 % of saturation with ammonium sulphate contained the whole of the antitoxin of the original plasma, those between 33 and 44 % of saturation contained 95 % of the antitoxin, while only 65 % was associated with the fraction isolated between 36 and 44 % of saturation with the salt.

Hence, notwithstanding the increased precipitation of antitoxin with the First Fraction Precipitates induced by the raising of the lower limit of saturation with ammonium sulphate to 33 and 36 % respectively, the proportionally greater removal of protein thereby effected led to an increase in the degree of concentration shown by the end products. But, in no case was the concentration increased beyond that which would have been obtained between the wider limits of precipitation had the serum been subjected to the preliminary adjustment recently advocated [Homer, 1918, 2].

These observations show that the cloudy end products of unadjusted sera can be avoided by precipitating the First Fraction at 33 or 36 % of saturation with ammonium sulphate. Such an expedient will probably appeal to those engaged in routine work as being a simpler process than the preliminary adjustment of the serum. However, for the reason given above (p. 49), those who adopt the procedure must be prepared to face losses considerably greater than were recorded in my previous communication [Homer, 1918, 2], where the First Fraction was precipitated at 30 % of saturation with the sulphate.

It is thus evident that, in the routine concentration of sera, in which a complete recovery of antitoxin is to be desired, the Second Fraction should not be precipitated within narrower limits than 30 to 44 % of saturation with ammonium sulphate irrespective of whether the serum has been suitably adjusted previous to its being heated.

II. IS THE ANTITOXIN EVENLY DISTRIBUTED THROUGHOUT THE PROTEIN FRACTIONS SUCCESSIVELY PRECIPITATED AT STAGES BETWEEN THE LIMITS FIXED IN (I)?

The Second Fraction Precipitates from heat-denaturated sera were further fractionated in successive stages between the limits suggested in (I) in order to ascertain whether the antitoxin was evenly distributed throughout the protein fractions respectively isolated, or whether it was mainly precipitated at a definite concentration with ammonium sulphate.

The consideration of these points was of importance for, in the former case, the evidence would indicate that, in order to isolate antitoxin as a separate entity, means other than the fractional precipitation of the serum with salts must be employed. In the latter case then, for special clinical purposes such as for the intravenous or intrathecal injection of antitoxin, where a high unitage of antitoxin per gram of protein would be advantageous, it might be advisable to precipitate the antitoxin and associated protein within the narrow limits thus indicated, even though by so doing the percentage recovery of the antitoxin were considerably reduced.

Experimental work was accordingly instituted in regard to the successive fractionation of the end products from the concentration of sera by the Homer (1916, 1918) methods. The investigation was confined to the examination of:

(a) *Clear End Products*, i.e. those from sera in which the reaction had been adjusted so as to ensure a heat-denaturation of about 35 %.

(b) *Cloudy End Products*, i.e. those from sera in which the heat-denaturation was of the order of 25 % or less.

In each case the end product was diluted with normal saline so as to reduce the protein content of the liquid to the order of 5 %.

To a known volume of the diluted liquid was added a sufficient volume of a saturated solution of ammonium sulphate to bring the concentration of this

salt in the mixture up to 30 % of saturation. If a precipitate were formed at this stage the liquids were filtered. To an aliquot part of the filtrate was added the volume of the saturated solution necessary to raise the concentration of the sulphate in the filtrate to 33 % of saturation. The liquids were again filtered and by a similar method of procedure the ammonium sulphate content of the filtrates was successively raised to the order of 36, 40, 45 and 50 % of saturation.

The precipitates isolated at each of the successive stages were pressed and dialysed in the usual way. The protein content of the respective residues from dialysis was estimated with the aid of the Zeiss Refractometer; determinations were also made of the percentage of the total antitoxin units appearing in the respective end products.

Owing to the scarcity of experimental animals the research could not be carried out so extensively as originally planned. Nevertheless the results, so far as could be obtained, have provided sufficient data to elucidate the problem under discussion.

(a) *The successive fractionation of the Clear End Products obtained from sera showing a heat-denaturation of about 35 %.*

Antidiphtheritic plasma was concentrated by the Homer (1918) method, the heat-denaturation of the plasma being regulated by the preliminary adjustment of the reaction.

The end products, obtained from the dialysis of the protein precipitated from the heated plasma between 30 and 45 % of saturation with ammonium sulphate, were clear and remained clear on dilution, for they were free from any opalescent suspension of heat-denaturated protein; subsequent tests showed that they were also free from "salt-insoluble" protein.

The end product, under consideration, contained 15,000 units of antitoxin per gram of protein. After the necessary dilution with saline it was precipitated in the manner described above; the results are embodied in Table I.

Table 1. *The successive fractionation of the Clear End Products from the concentration of antitoxic sera in which a heat-denaturation of 35 % had been induced.*

(The end product taken contained 15,000 units of antitoxin per gram of protein.)

Percentages of saturation with ammonium sulphate marking the limits for the precipitation of the various fractions	The residues from the dialysis of the respective fractions showed			
	Appearance	Percentage of the total proteins	Percentage of the total antitoxic units	Number of units of antitoxin associated with 1 g. of protein
0-30	—	0.0	0.0	—
30-33	clear	traces	traces	—
33-45	clear	80.0	73.5	13,800
33-40	clear	62.5	59.5	14,500
40-45	clear	18.5	16.5	13,800
45-50	clear	2.5	2.8	16,000

From a study of the table it will be seen that there was no appreciable precipitation of protein when the concentration of ammonium sulphate in the diluted end product was successively raised to 30 and to 33 % of saturation respectively.

Between 33 and 40 % of saturation with the sulphate there was precipitated 62.5 % of the total pseudoglobulin of the end product, and with this protein there was associated 59.5 % of the total antitoxin.

The protein precipitated from the diluted end product between 33 and 45 % of saturation with ammonium sulphate comprised 80 % of the total protein and 73.5 % of the total antitoxic units. Further fractionation indicated that 62.5 % of the protein and 59.5 % of the antitoxin were precipitated between 33 and 40 % of saturation with ammonium sulphate; 18.5 % of the protein and 16.5 % of the antitoxin were precipitated between 40 and 45 % of saturation with the sulphate; 2.5 % of the total protein and 2.8 % of the total antitoxin were precipitated between 45 and 50 % of saturation with the sulphate.

Unfortunately owing to the shortage of experimental animals the fractionation was not continued further.

An examination of these results shows that, within the limits of experimental error, the percentage precipitation of antitoxin in the respective sub-fractions is directly proportional to the percentage precipitation of protein. Moreover, it will be seen that, at each stage of the precipitation, the load of antitoxin per gram of protein in solution was practically the same as that exhibited in the original liquid taken for fractionation, viz. of the order of 15,000 units.

Similar results were obtained from the fractionation of other clear end products.

The data thus obtained have furnished sufficient evidence for the conclusion that the antitoxin precipitated with its associated proteins from the heat-denaturated sera under discussion is proportionately distributed between the protein fractions successively precipitated at increasing concentrations of ammonium sulphate.

(b) The successive fractionation of the Cloudy End Products from sera showing a heat-denaturation of 25 % or less.

It has been demonstrated that the concentration of antitoxic sera which, prior to their being heated, contained an unsuitable percentage of phenol and its homologues or of which the reaction lay between P_H 5.5 and 7.4, led to the production of cloudy end products. The latter yield a lower degree of concentration than that furnished by sera adjusted so as to ensure not only a more extensive denaturation but also better conditions for the precipitation of the heat-denaturated protein with the First Fraction Precipitates at 30 % of saturation with ammonium sulphate.

Some of these cloudy products were taken and fractionally precipitated in stages in the manner described above (p. 50).

The data with regard to the percentage precipitation of the total protein and of the total antitoxin with the fractions precipitated at the various stages have been included in Table II.

Table II. *The successive fractionation of the Cloudy End Products from the concentration of antitoxic plasma in which a heat-denaturation of 25 % had been induced.*

(The end product under consideration contained 12,000 units of antitoxin per gram of protein in solution.)

Percentages of saturation with ammonium sulphate marking the limits for the precipitation of the respective fractions	The residues from the dialysis of the various protein fractions showed			
	Appearance	Percentage of the total protein	Percentage of the total antitoxin	Number of units of antitoxin associated with 1 g. of protein
0-30	—	0.0	0.0	—
30-33	thick suspension	16.0	6.0	4,550
30-36	markedly opalescent	48.8	35.0	8,600
33-36	clear	32.0	28.0	10,500
33-40	clear	57.0	58.0	12,300
40-45	clear	8.9	10.0	13,500
36-40	clear	24.5	30.0	14,500

In the case recorded, the addition of ammonium sulphate to the diluted end product to the extent of 30 % of saturation did not cause a precipitation of protein.

Between 30 and 33 % of saturation there was precipitated 16 % of the total protein and with this was associated only 6 % of the total antitoxin. The fraction between 30 and 36 % of saturation contained 48.8 % of the total protein and 35 % of the total antitoxin. The percentage of the total protein precipitated in both of these fractions is considerably greater than that of the antitoxin, the disparity being more marked in the former fraction.

An analysis of the respective results shows that, by difference, there was a direct proportionality between the percentage of protein and antitoxin in the fraction precipitated between 33 and 36 % of saturation with the sulphate. A separation of the protein precipitated at 33 % of saturation with the sulphate was made and the filtrate was sub-fractionated further, the details being given in Table II.

These results indicate that, after the preliminary separation of the protein precipitated at 33 % of saturation with the sulphate, the sub-fractions successively isolated show the same proportionality as regards the relative precipitation of antitoxin and protein as was found in the sera discussed in (I).

In another case, not recorded in the Table, in which the heat-denaturation was less than 20 %, the sub-fraction precipitated between 30 and 33 % of saturation with ammonium sulphate contained about 20 % of the total protein

and about 5 % of the total antitoxin; that between 33 and 36 % of saturation with the sulphate contained 36 % of the total protein and about 16 % of the total antitoxin. In such sera the sub-fractions isolated from the filtrates after the preliminary removal of the protein precipitated at 36 % of saturation showed the proportionality exhibited after the preliminary removal of protein precipitated at 33 % in the previous case and at 30 % of saturation in the sera discussed in (a).

A study of Table II also shows that in the First Sub-fraction (30–33 % of saturation) the load of antitoxin per gram of protein is considerably less than in the subsequent fractions and in the original solution. Moreover, as was to be expected, the sub-fractions subsequently precipitated after the preliminary removal of this First Sub-fraction contained a load of antitoxic units per gram of protein slightly greater than that shown by the original liquid. However, the load of antitoxin per gram of protein was in no case greater than it would have been had the reaction been suitably adjusted as in (a).

From these results it is obvious that in heat-denaturated sera showing a denaturation of 25 % the concentration of the end products and their degree of clarity can be increased by raising the precipitation limit for the First Fraction Precipitates to 33 % of saturation with ammonium sulphate. In sera showing a lower degree of denaturation it is necessary to raise the limit to 36 % of saturation in order to achieve the maximum advantage. In these cases the increased concentration of the sulphate ensures the precipitation and separation of heat-denaturated globulin to which a small portion only of the antitoxin is attached: this protein would have been precipitated at 30 % of saturation in sera suitably adjusted as in (a).

After the preliminary removal of the denaturated globulin to which a comparatively low percentage of the antitoxin is attached, the remainder of the antitoxin was found to be evenly distributed throughout the successive sub-fractions precipitated by successively raising the concentration of ammonium sulphate.

It was also observed that, in the unadjusted sera under discussion, the protein fractions respectively precipitated between 30 and 33, and between 30 and 36 % of saturation with the sulphate did not dialyse completely. The increased concentration of sulphate had induced an apparent irreversible precipitation of heat-denaturated protein. The precipitate thus formed was only partially redissolved by long saturation in about ten times its bulk of a saturated solution of salt.

A study of the data given in Tables I and II shows that, while from the whole serum the proteins associated with the antitoxin were precipitated between 30 and 45 % of saturation with ammonium sulphate, the precipitation of the refined products within the same limits yielded only 80 % of the protein-antitoxin combination. It has been my experience that the percentage addition of ammonium sulphate necessary for the complete precipitation of a particular fraction of an individual protein is influenced by the concentration

of the latter and by the presence of other proteins in solution; it is also affected by the degree of purity of the protein and its rarity in solution. The relationships hereby involved are being more fully investigated.

SUMMARY.

1. For the complete recovery of antitoxin during the concentration of sera, showing a heat-denaturation of 35 % or less, by fractional methods employing the use of ammonium sulphate it is advisable to precipitate the Second Fraction between 30 and 45 % of saturation with the sulphate.

If the upper limit be reduced there will be incomplete precipitation of pseudoglobulin and antitoxin and a certain percentage of the latter will be discarded with the "albumin" filtrates.

If the lower limit be raised then antitoxin will be precipitated with the First Fraction Precipitates in a form not readily soluble in brine, and, therefore, to all intents and purposes lost.

2. In the precipitation of the Second Fraction from sera in which a denaturation of 25 % or less has been induced, the raising of the lower limit to 33 or to 36 % of saturation leads to the production of clearer and more concentrated end products than those obtained by the adoption of the lower limit of 30 % of saturation.

However, for the reasons given in (1) the benefit thus gained is minimised by the greater losses of antitoxin thereby incurred.

Moreover, the degree of concentration is not increased beyond that which would have been obtained had the serum been suitably adjusted and subsequently treated as in (1).

3. In heated sera showing a denaturation of 35 % or less the bulk of the antitoxin is associated with the proteins precipitated between 36 and 45 % of saturation with ammonium sulphate.

4. The further fractionation of the protein isolated between the limits indicated in (3) showed that the percentage of the total antitoxin precipitated between progressively increasing percentages of saturation with the sulphate is directly proportional to the percentage precipitation of protein at the respective stages.

5. From these observations it is clear that, in order to isolate antitoxin, means other than the fractional precipitation of the serum proteins must be employed.

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VII. ON THE INCREASED PRECIPITABILITY OF PSEUDOGLOBULIN AND OF ITS ASSOCIATED ANTITOXIN FROM HEAT-DENATURATED SOLUTIONS.

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SOME time ago an investigation was undertaken in order to furnish data as regards the increased precipitability of pseudoglobulin relatively to that of its associated antitoxin from solutions of which, prior to their being heated, the reaction had been adjusted to values between P_H 4.5 and 9.5.

In view of the need for the immediate application of the results to routine work, the data obtained with regard to the precipitability of the protein from solutions of which the reaction had been adjusted to about P_H 8.0 were included in a previous paper [Homer, 1917, 1]; the data furnished from the wider aspect of the problem form the subject matter of the present communication.

The study of the changes in the precipitability of the pseudoglobulin presented no difficulty, but, unfortunately, the scarcity of experimental animals has considerably limited the scope of the investigation as regards the fate of the antitoxin.

(a) THE INCREASED PRECIPITABILITY OF PSEUDOGLOBULIN FROM ITS HEAT-DENATURATED SOLUTION BY AMMONIUM SULPHATE.

The solution of pseudoglobulin and antitoxin used in the experiment was prepared as follows from unheated oxalated antidiphtheritic plasma pooled from the bleedings from several horses.

To 4 litres of plasma was added an equal volume of a saturated solution of ammonium sulphate; the mixture was allowed to remain at room temperature for 4 hours before being filtered. The precipitate, after being well drained, was thrown into 8 litres of a half saturated solution of ammonium sulphate, care being taken both to break up the lumps of precipitate and to keep the mixture stirred from time to time. After two days of this treatment the mixture was filtered. The well drained precipitate was gently pressed between cloths and boards to express as much of the adhering fluid as possible; it was then dissolved in 4 litres of water and the solution was submitted to a repetition of the above described process.

To the solution of globulins finally obtained were added 4 litres of a saturated solution of sodium chloride together with sufficient solid salt to ensure complete saturation. The brined liquid was allowed to remain at room temperature for seven days and was then filtered. To the filtrate, containing the "salt-soluble" globulin in solution, was added 0.25 % of glacial acetic acid; the ensuing precipitate of pseudoglobulin was filtered, pressed and dialysed in the usual way. The residue from dialysis was diluted so as to reduce the protein content to the same order as that of the original plasma.

To twelve separate volumes of the experimental liquid were added varying amounts of acetic acid or of ammonia to adjust the reaction to values between P_H 4.5 and 9.5; the $[H^+]$ of each of the liquids was measured by the electrical method. The stoppered bottles containing the adjusted liquids were placed in a water bath at 57.5° and were kept at this temperature for a period of six hours.

Samples of the unheated solutions were made respectively 26, 30, 33, 36, 38, 40, 42.5, 44.5 and 47 % of saturation with ammonium sulphate and were then filtered. The protein content of the filtrates was measured by means of the Zeiss Immersion Refractometer, and the data thus obtained were compared with those derived from similar determinations of the heated liquids.

Table I. *Showing the influence of heat-denaturation on the precipitability of pseudoglobulin from its solutions at increasing concentrations of ammonium sulphate.*

The solutions which contained 7.48 % of pseudoglobulin were heated at 57.5° for 6 hours.

Experimental liquid No.	P_H of the liquids prior to their being heated	Residual percentage of pseudoglobulin in the filtrates from the heated liquids respectively brought to the following degrees of saturation with ammonium sulphate								
		26	30	33	36	38	40	42.5	44.5	47
0 control	unheated	7.40	7.33	5.16	2.48	1.88	1.48	1.04	0.40	0.20
1	9.5	3.47	3.08	1.37	0.60	0.20	0.00	0.00	0.00	0.00
2	9.3	4.38	3.61	1.74	0.64	0.34	0.14	0.00	0.00	0.00
3	8.7	7.04	4.31	2.14	1.40	0.53	0.36	0.00	0.00	0.00
4	8.3	7.36	4.68	2.58	1.64	1.26	1.05	0.21	0.00	0.00
5	7.5	7.48	5.33	3.24	1.54	1.50	—	0.15	0.02	0.00
6	6.4	7.13	5.64	3.56	2.47	1.22	0.75	0.21	0.02	0.00
7	5.9	7.17	5.46	3.18	2.28	1.26	0.80	0.20	0.00	0.00
8	5.1	5.05	4.62	2.95	1.79	0.95	0.75	0.21	0.00	0.00
9	4.9	3.36	2.77	1.87	0.18	0.20	0.00	0.00	0.00	0.00
10	4.65	1.85	1.85	1.62	0.00	0.00	0.00	0.00	0.00	0.00
11	4.5	1.05	1.05	0.46	0.00	0.00	0.00	0.00	0.00	0.00

The results embodied in Table I indicate the percentages of pseudoglobulin remaining in solution in the filtrates from the respective pseudoglobulin-ammonium-sulphate mixtures. From these data was calculated the percentage precipitation of pseudoglobulin at the respective stages: the latter results have been depicted in Table II.

Table II. *Showing the relation between the extent of the heat-denaturation of pseudoglobulin and its increased precipitability by ammonium sulphate.*

The solutions which contained 7.48 % of pseudoglobulin were heated at 57.5° for 6 hours.

Experi- mental liquid No.	P_H of the liquids prior to their being heated	Percentage denaturation of the pseu- doglobulin	Percentage of the total pseudoglobulin precipitated from the solutions by the addition of ammonium sulphate to the following degrees of saturation								
			26	30	33	36	38	40	42.5	44.5	47
0 control	unheated	—	0.0	1.6	30.1	67.0	75.7	80.0	86.0	94.7	97.3
1	9.5	53.5	53.4	58.8	82.4	92.0	97.3	100.0	100.0	100.0	100.0
2	9.3	47.3	41.2	51.5	76.4	92.5	95.7	98.1	100.0	100.0	100.0
3	8.7	38.1	5.5	42.1	71.3	80.1	92.9	95.9	100.0	100.0	100.0
4	8.3	34.0	1.0	37.6	62.7	78.0	84.0	86.0	97.3	100.0	100.0
5	7.5	25.0	0.0	27.5	56.2	79.3	80.0	—	98.0	100.0	100.0
6	6.4	21.8	3.0	24.3	51.0	67.0	84.0	90.0	98.0	99.0	100.0
7	5.9	22.7	3.1	26.4	55.6	69.4	84.2	89.0	97.5	99.0	100.0
8	5.1	33.3	32.2	38.0	60.5	76.0	87.2	89.5	97.5	100.0	100.0
9	4.9	60.0	55.0	63.0	75.7	98.0	98.0	100.0	100.0	100.0	100.0
10	4.65	70.0	75.5	75.5	78.0	100.0	100.0	100.0	100.0	100.0	100.0
11	4.5	83.0	86.0	86.0	94.0	100.0	100.0	100.0	100.0	100.0	100.0

From a study of the tables it will be seen that, in the unheated liquid, the precipitation of pseudoglobulin begins at about 28 % and is not quite completed at 47 % of saturation with the sulphate.

In the heated liquids there was complete precipitation of the protein at a much lower concentration of sulphate than that required in the unheated liquid. Thus, where a heat-denaturation of from 20 to 30 % had been induced, the precipitation of protein was complete at about 44 % of saturation with the sulphate; where the denaturation was of the order of 50 % there was complete precipitation of the protein between 38 and 40 % of saturation with the sulphate; as the denaturation was further increased, so the concentration of ammonium sulphate necessary for the complete precipitation of the pseudoglobulin diminished.

It will also be seen that the precipitability of the protein, at each degree of saturation with the sulphate, was considerably greater in the heated than in the unheated liquids, the increase being most pronounced where the heat-denaturation was greatest.

The latter point is more clearly brought out by the plotting of the results given in Table II in the form of curves. For the sake of clearness it was necessary to present the curves in two figures. In Fig. 1 have been included the curves representing the precipitation of protein by ammonium sulphate from the unheated liquid and from the heated acid liquids of which the reaction lay between the values P_H 7.4 and 4.5: the curves in Fig. 2 represent the precipitation of protein from the heated alkaline liquids of which the reaction lay between P_H 7.1 and 9.5.

From the relative positions of corresponding points on the curves it is evident that, at the respective concentrations of ammonium sulphate, the

precipitation of protein is greater from the heated than from the unheated liquids.

To take a case in point: in the unheated solution 40 % of protein was converted from the emulsoid to the suspensoid condition at 34 % of saturation with ammonium sulphate: the same percentage conversion was obtained in the heated liquids at lower concentrations with ammonium sulphate. Thus, in the heated liquids Nos. 1, 2, 3, 4, 5, 6, 7 and 8 in which the denaturation had been of the order of 53.5, 47.3, 38.1, 34, 25, 21.8, 22.7 and 33.3 % the above precipitation of protein was respectively achieved at 25, 28.8, 30.5, 31, 31.5, 31.5, 31 and 30 % of saturation with the sulphate.

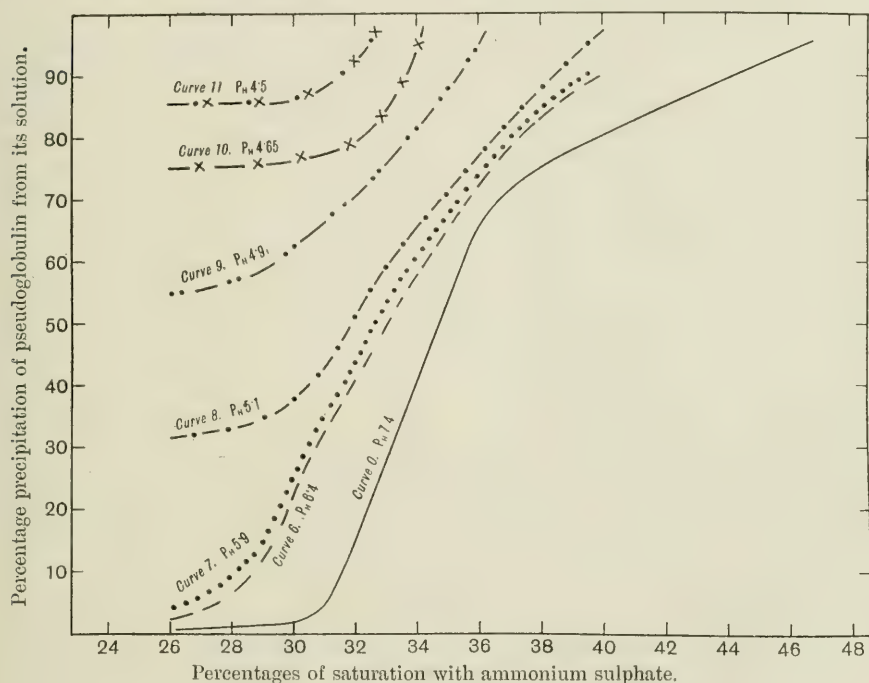


Fig. 1.

Curves representing the percentage precipitation of pseudoglobulin from unheated and from heated solutions by increasing concentrations of ammonium sulphate.

Curve 0 for the unheated liquid; Curves 6, 7, 8, 9, 10 and 11 for the heated liquids of which the reaction, prior to their being heated, had been adjusted to P_H 6.4; 5.9; 5.1; 4.9; 4.65 and 4.5 respectively.

It will be seen that the group of curves 0, 3, 4, 5, 6 and 7, representing the precipitation of protein from those liquids in which a heat-denaturation of 40 % or less had been induced, is marked by a similarity of configuration throughout the whole of the range investigated; the shift in the relative positions of corresponding points on the curves is constant and is a measure of the increased precipitation induced by the heat-denaturation of the protein.

On the other hand, while there is a marked similarity between the con-

figuration of the individual curves in the group 1, 2, 8, 9, 10 and 11 the composite type of curve differs from that of the former group. The shift in the relative positions of corresponding points is not constant, the magnitude of the increased precipitation being relatively greater for the lower concentrations with the sulphate, a phenomenon which is accompanied by an increasing "salt-insolubility" of the denaturated protein precipitated at stages in the flat portion of the curves.

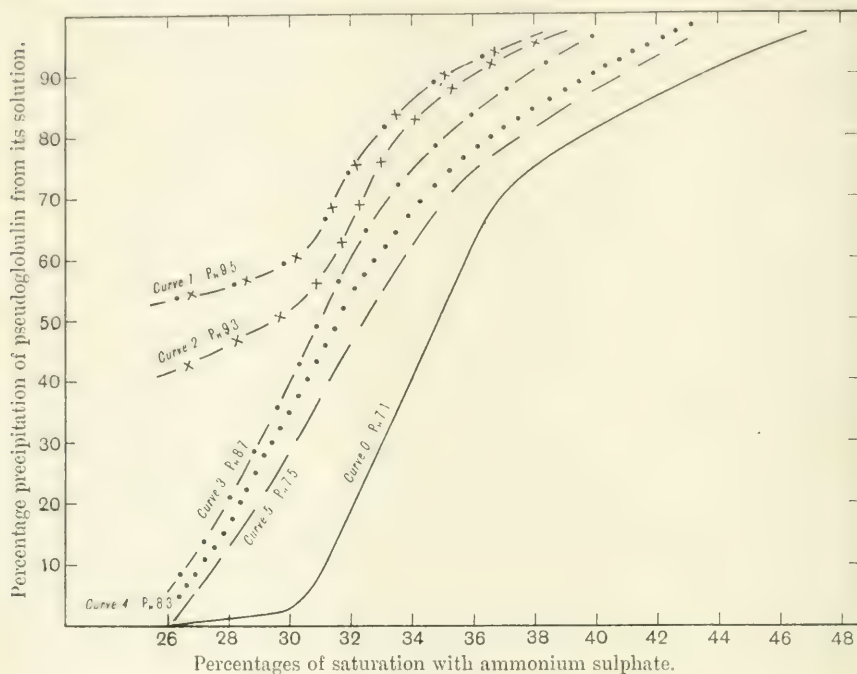


Fig. 2.

Curves representing the percentage precipitation of pseudoglobulin from unheated and from heated solutions by increasing concentrations of ammonium sulphate.

Curve 0 for the unheated solution. Curves 1, 2, 3, 4 and 5 for the heated solutions of which, prior to their being heated, the reaction had been adjusted to P_H 9.5; 9.3; 8.7; 8.3 and 7.5 respectively.

(b) THE PRECIPITATION OF ANTITOXIN WITH HEAT-DENATURATED PSEUDOGLOBULIN.

It had been my intention to ascertain the limits of saturation with ammonium sulphate between which the antitoxin was precipitated from each of the twelve solutions dealt with in (a). But, owing to the long continued scarcity of experimental animals, it was impossible to carry out the scheme on such an extensive and comprehensive scale, and, therefore, the scope of the work has been restricted to a determination of the extent of the association of antitoxin with the protein precipitated at a conveniently chosen concentration with ammonium sulphate, viz. at 30 % of saturation with the salt.

PRECIPITABILITY OF PSEUDOGLOBULIN AND ANTITOXIN 61

The method of procedure was as follows. To each of the twelve solutions dealt with in (a) was added the necessary volume of a saturated solution of ammonium sulphate to bring the concentration of the latter in the mixture up to 30 % of saturation. The mixture was filtered; the protein and the antitoxin content of the filtrates were estimated in the usual way; the values obtained were compared with those for the experimental solution. From these data was calculated the percentage of the total antitoxin precipitated with the protein from the respective solutions.

The data thus furnished were incorporated in Table III and in the curve in Fig. 3. They show that while in the unheated liquid the precipitation of antitoxin at 30 % of saturation with the sulphate is negligible, in the heated liquids the proportion precipitated at this stage is considerable and increases with the extent of the denaturation.

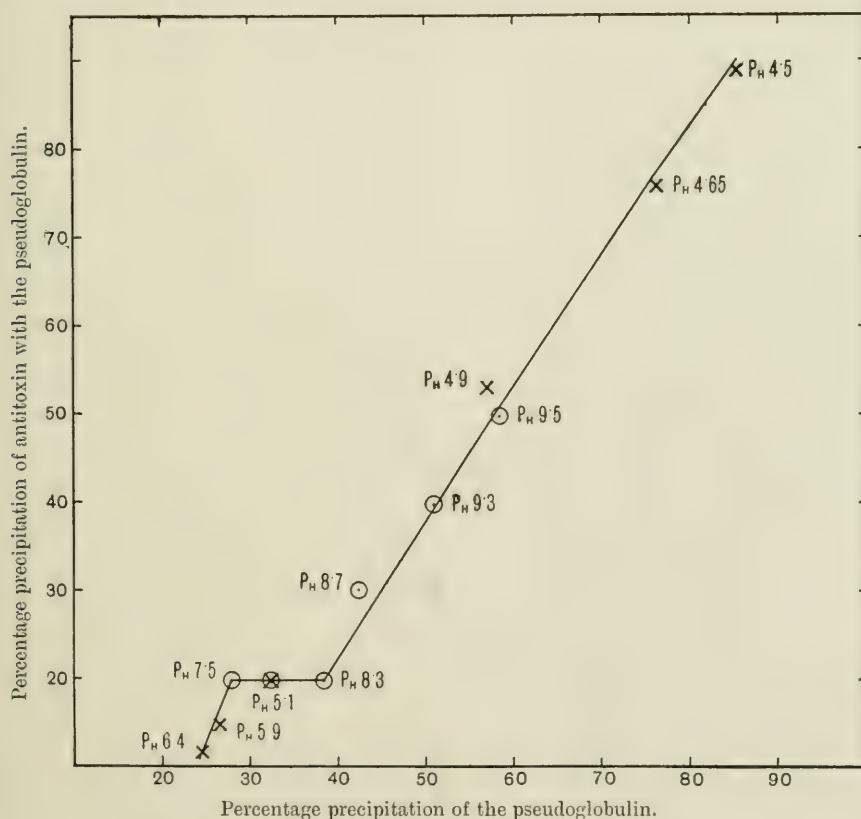


Fig. 3.

Curve representing the relative precipitations of pseudoglobulin and antitoxin at 30 % of saturation with ammonium sulphate from solutions of which, prior to their being heated, the reaction had been adjusted to values between P_H 4.5 and 9.5.

Table III. *Showing the relation between the extent of the heat-denaturation of pseudoglobulin and the precipitation of antitoxin with the denaturated protein by 30 % of saturation with ammonium sulphate.*

The solution of pseudoglobulin showed a potency of 450 units per cc.

Experimental liquid No.	P _H of the liquid prior to its being heated	Percentage denaturation of the pseudoglobulin	Percentage of the total pseudoglobulin precipitated at 30 % of saturation with ammonium sulphate	Percentage of the total antitoxic units precipitated with the pseudoglobulin at 30 % of saturation with ammonium sulphate
0 control	7.1	unheated	1.6	—
1	9.5	53.5	58.8	50
2	9.3	47.3	51.5	40
3	8.7	38.1	42.1	30
4	8.3	34.0	37.6	20
5	7.5	25.0	27.5	20
6	6.4	21.8	24.3	12
7	5.9	22.7	26.4	15
8	5.1	33.3	38.0	20
9	4.9	60.0	63.0	55
10	4.65	70.0	75.5	> 66 < 80
11	4.55	83.0	86.0	> 80 < 90
12	4.4	too solid to measure		> 90

The precipitation of antitoxin was least from those liquids showing the least extensive denaturation (Nos. 4, 5, 6, 7 and 8), and in these cases the proportion of the total protein precipitated by the sulphate was not a linear measure of the proportion of the total antitoxin associated with the precipitate. In the more acid and in the more alkaline liquids, there was a marked increase in the precipitation of protein and of antitoxin, but it was found that the increased precipitation of protein beyond that shown in Nos. 4, 5, 6, 7 and 8 was a measure of the accompanying increased precipitation of antitoxin at this stage.

It seems justifiable to conclude that *in the unheated liquid* there is a comparatively small load of antitoxin attached to the fraction of pseudoglobulin which is precipitated at about 34 to 35 % of saturation with ammonium sulphate, that is, with the pseudoglobulin which, in unheated sera, would be precipitated at concentrations of ammonium sulphate slightly greater than that required for the precipitation of the euglobulin. A comparatively low degree of heat-denaturation suffices to ensure the precipitation of this fraction of the pseudoglobulin at 30 % of saturation with the sulphate. A more extensive denaturation leads to the precipitation at this stage of fractions of the pseudoglobulin which, in the unheated liquids, would require considerably greater concentrations of the sulphate. The antitoxin is evenly distributed throughout the range of the latter fractions, a deduction readily made from the study of the curve in Fig. 3 taken in conjunction with the results obtained in a recent investigation of the further fractionation of the proteins of heat-denaturated sera [Homer, 1919].

The data thus obtained also furnish additional evidence that, in the fractional precipitation of antitoxin and pseudoglobulin by ammonium sulphate, an increase in the extent of the denaturation, up to a point, favours the isolation of the antitoxin with a correspondingly decreased percentage of the original protein. But beyond this previously described limit [Homer, 1917, 2, 3; 1919] a more extensive denaturation ceases to be advantageous, for, with the further increased removal of protein there is a correspondingly increased proportional precipitation of antitoxin; moreover, much of the antitoxin thus precipitated is lost, as the protein with which it is associated has been converted into a "salt-insoluble" condition.

The consideration of the above results taken in conjunction with those from my previous investigations leads me to the conclusion that, in order to isolate the bulk of the antitoxin from antitoxic sera in association with a minimum percentage of the total serum proteins, there is no need for the preliminary prolonged heating of the serum originally advocated by Banzhaf and Gibson [1907] and now generally adopted by those engaged in the concentration of sera. For while I have shown that, if the preliminary heating be conducted to the best advantage, the antitoxin can be isolated from *heated* sera in association with a minimum amount of protein in the fraction precipitated between 30 and 44 % of saturation with ammonium sulphate, my more recent work demonstrates that the same results would have been obtained by the isolation of the protein fraction precipitated from the *unheated* serum between (*circ.*) 36 and 50 % of saturation with the sulphate.

The concentration of antitoxic sera by the fractional precipitation of the unheated serum by ammonium sulphate, as indicated above, is obviously a shorter process than those in which a preliminary heating of the serum is adopted. Furthermore, the fractional precipitation of the unheated plasma or serum between the higher limits for the concentration of ammonium sulphate is not attended with the filtration difficulties so often experienced with heated sera fractionated between the lower limits, for, in the former case not only is the complete separation of the euglobulin with the First Fraction assured, but the possibility of trouble arising from the incomplete agglutination of particles of heat-denaturated protein is avoided.

The above considerations seem to indicate that from the point of view of the isolation of antitoxin and its associated protein, there is no practical advantage to be gained by the preliminary heat-denaturation of the serum.

There is, however, some evidence to show that, from a clinical point of view, the heating of the serum is beneficial. For, it has been found that the subcutaneous injection into mice and guinea-pigs of a given amount of cresylic acid causes more pronounced toxic symptoms when administered in solution in presence of unheated serum proteins than when in the presence of serum proteins previously heated to 57.5° for four hours.

My observations with regard to the fractional precipitation and concentration of unheated sera and to the lessened toxicity of cresylic acid in presence of heat-denaturated proteins will be dealt with in later communications.

SUMMARY.

1. The increased precipitability of pseudoglobulin from its heat-denaturated solutions at concentrations of ammonium sulphate ranging from 26 to 47 % of saturation is a function of the heat-denaturation.

2. The increased precipitation of pseudoglobulin thus induced in (1) at 30 % of saturation with ammonium sulphate is accompanied by an increased precipitation of antitoxin. With the least extensive denaturation, the percentage of the total proteins precipitated at this stage is greater than that of the antitoxin; as the denaturation increases so the further increased precipitability of the protein becomes a linear measure of the increased precipitation of the antitoxin.

3. In the concentration of antitoxic sera by the fractional precipitation of the serum with ammonium sulphate there is no need for a preliminary prolonged heating of the serum. The results that are now obtained by the isolation from the heated serum of the protein fraction precipitated between 30 and 44 % of saturation with ammonium sulphate could be obtained from the *unheated* serum between 36 and 50 % of saturation with the sulphate.

4. The heating of the serum reduces the toxicity of the cresylic acid-protein complex.

5. The data furnished in this research also point to the conclusion that, in order to isolate antitoxin as a separate entity, means other than the fractional precipitation of pseudoglobulin solutions by salts must be employed.

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VIII. THE RELATION OF SUGAR EXCRETION TO DIET IN GLYCOSURIA.

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THE modern treatment of diabetes is founded on the experimental work of Allen [1915] on the effects of starvation on the excretion of sugar in glycosuria. The observation that the urine of many people suffering from this disease may be freed from sugar by starvation forms the basis of the experimental work detailed in the following pages.

It is clear that if the urine of a man suffering from glycosuria is rendered sugar free by starvation, then, the effect of any diet on his sugar excretion may be determined. The accuracy of the results is limited by the fact that it is not desirable to keep a man in a constant state of starvation. But, by a careful choice of the time at which a desired food is given, and by making a graphic time record of the sugar subsequently excreted, a considerable amount of information may be obtained on the effect of that foodstuff on the excretion of sugar. From an analysis of these results, considered in conjunction with other observations in this disease, an hypothesis has been advanced on the causation of glycosuria in *diabetes mellitus*.

The main results recorded were obtained from a man, aet. 53, suffering from *diabetes mellitus*. When he came under observation he was excreting, *per diem*, about 250 g. of dextrose contained in four litres of urine.

The sugar determinations were made by a method originally described by Wood and Berry [1903]. In this method 25 cc. of a copper solution (suggested by Soldaini [1876]) were boiled for 15 minutes with 1 cc. of urine, and the precipitated cuprous oxide was filtered off by asbestos felt contained in a Gooch crucible. The cuprous oxide was dissolved in 25 cc. of a 2.5 % solution of ferric sulphate contained in 25 % H_2SO_4 , and the equivalent amount of ferrous sulphate formed was estimated by a standard solution of permanganate. The strength of the permanganate solution was such that 1 g. of dextrose was equivalent to 264 cc. KMnO_4 .

The man's diet was carefully weighed and controlled during the experimental period. Whenever he emptied his bladder the amount of urine excreted and the time of excretion were noted. The quantity of dextrose contained in 1 cc. of urine was determined. The man was encouraged to micturate at frequent intervals so that a time curve showing the rate of sugar excretion could be obtained with a fair degree of accuracy.

(a) *Starvation.* When the man first came under observation a three days' fast was required to free his urine from sugar. The following detailed experiment was made three weeks later and shows, *inter alia*, that a considerable improvement had taken place in his condition. His last meal was taken at 6.30 p.m. Within 24 hours his urine was free from sugar. The table shows the effect of deprivation of all food on the average sugar and urine excretion per hour.

Time	Amount of urine cc.	cc. KMnO_4 for 1 cc.	Total sugar g.	Sugar per hour g.	Urine per hour cc.
4.30 p.m.	253	16.4	15.7	—	—
8 "	224	12.6	10.7	3.6	64
Midnight	337	8.4	10.8	2.68	84
2.30 a.m.	280	9.9	10.5	4.2	112
8 "	224	7.9	6.7	1.22	40
9 "	224	7.95	6.73	6.73	224
11 "	450	1.35	2.3	1.15	225
1.30 p.m.	476	0.85	1.54	0.62	190
3.30 "	253	0.25	0.24	0.12	226
5.30 "	364	0.05	0.068	0.034	182
8 "	503	0.05	0.096	0.038	201
1 a.m.	224	0.05	0.042	0.008	45

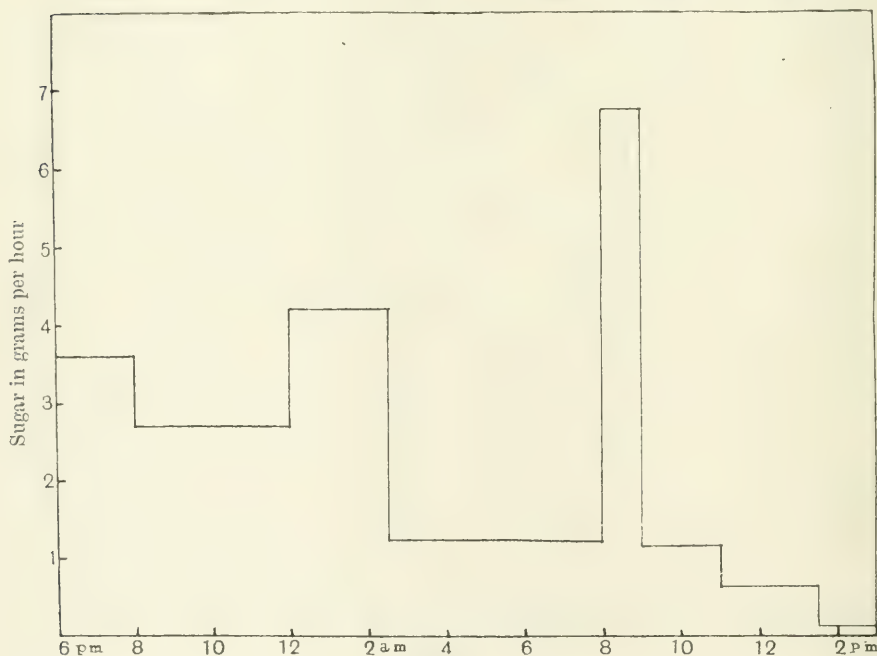


Fig. 1.

The results are shown graphically in Fig. 1. The rapid disappearance of sugar from the urine in starvation is shown in a clear way. From midnight to noon he excreted 37 g., from noon to midnight 2 g. of dextrose. The disappearance of sugar from the urine when no food was taken afforded a means

whereby the effect of diet on the excretion of dextrose in the urine could be determined. Incidentally it may be remarked that the curve shows the effect of taking caffeine (contained in tea without milk or sugar) at 8 a.m. The amount of urine was greatly increased, and, although the percentage of sugar in the urine remained constant, yet the total quantity of sugar excreted showed a prominent rise. Again, it is well known that the amount of urine excreted in glycosuria is roughly proportional to the amount of sugar contained in the urine. It may be observed that whereas this generalisation held true for the first 12 hours when the urine contained a large amount of dextrose, the parallelism ceased when the urine became sugar free.

(b) *Extractives*. The object of this experiment was to ascertain whether the absorption of any substance from the alimentary canal, considered independently of its energy value, caused an excretion of sugar in the urine. Since only one-fifth of the total energy contained in the extractives of meat is utilised by the body, the man was given 840 cc. of beef tea to drink when his urine was free from sugar. The following table shows the results observed:

Time	Amount of urine cc.	cc. KMnO_4 for 1 cc.	Total sugar g.	Sugar per hour g.	Urine per hour cc.
9-10.15 a.m.	56	1.6	0.34	0.15	25
4.30 p.m.	140	6.6	3.58	0.7	25
8 ,,	84	4.6	1.45	0.41	24

The beef tea was taken between 2.30 p.m. and 4.30 p.m. Only a small quantity of sugar was excreted, about equal to the carbohydrate extracted from the beef. No definite evidence was obtained in favour of the assumption that the extractives contained in beef may give origin to sugar, nor that the act of absorption from the alimentary canal may cause the excretion of sugar in the urine.

(c) *Alcohol*. It was of interest to determine the effect of a relatively simple substance of a high calorific value on the excretion of sugar. Alcohol was chosen for this purpose since it has obtained a reputation as a foodstuff of considerable value in the dietetic treatment of *diabetes mellitus*. 112 cc. of whisky containing 30 % of alcohol were given in two portions at 10 p.m. and 4 a.m. respectively. In each case water was added to make the volume of the diluted fluid 250 cc. The following figures show the excretion of sugar and urine during the experimental period.

Time	Amount of urine cc.	cc. KMnO_4 for 1 cc.	Total sugar g.	Sugar per hour g.	Urine per hour cc.
8-11 p.m.	56	3.5	0.74	0.245	19
1 a.m.	112	3.0	1.3	0.65	37
2 ,,	140	0.8	0.42	0.42	140
6 ,,	56	0.9	0.20	0.05	14

The figures show that after the ingestion of alcohol the excretion of sugar increased a small amount only—from 0.245 g. to 0.65 g. of sugar per hour. Since alcohol is metabolised to a considerable extent by the body—only 2 %

being excreted—it is evident that the tissues can utilise a simple substance containing carbon, hydrogen and oxygen only without a concomitant excretion of sugar in the urine. This fact is of some practical value since the calorific value of alcohol is seven and the available energy obtained from 112 cc. of whisky containing 30 % alcohol amounts to 235 K.

(d) *Protein*. The effect of protein on the excretion of sugar was determined by giving a meal of 112 g. of caseinogen at 4.30 p.m. The urine was not free from sugar before giving the caseinogen since, four hours previously, the man had eaten a small quantity of beef and green vegetables. However, the comparative results, and particularly the graphic record of these results, clearly show the effect of caseinogen on the excretion of sugar.

Time	Amount of urine	cc. KMnO_4 for 1 cc.	Total sugar	Sugar per hour	Urine per hour
10.30 a.m. to	cc.		g.	g.	cc.
2.30 p.m.	196	2.1	1.56	0.39	49
4.30 „	280	3.6	3.8	1.9	140
8 „	168	4.5	3.12	0.89	45
10 „	336	4.9	6.25	3.12	168
5 a.m.	364	0.4	0.56	0.08	52

The results are shown graphically in Fig. 2. It is evident from the figure that the excretion of sugar between 8 p.m. and 10 p.m. was due to the ingested caseinogen. On this assumption the metabolism of 112 g. of caseinogen caused the excretion of 6 g. of dextrose in the urine. The caseinogen was sugar-free so that the dextrose must have originated from the amino-acids of the protein. It is of interest to compare this result with that obtained by Janney [1915] on a phlorhizinised dog. In this case 48 % of the ingested protein appeared in the urine as dextrose. Since it is assumed that a dog treated with this drug is incapable of utilising dextrose, the deduction is made that caseinogen contains 48 % of amino-acids which normally may give rise to dextrose. From these figures it may be calculated that the man was capable of utilising 89 % of the amino-acids of caseinogen which were available for conversion into dextrose.

(e) *Carbohydrate*. Whatever the carbohydrate eaten—starch, cane sugar, lactose or laevulose—dextrose only was excreted in the urine. The effect of carbohydrate on the excretion of sugar was determined in detail. Carbohydrate in three forms was given: (i) laevulose, (ii) cane sugar, (iii) oatmeal (starch).

(i) *Laevulose*. The view is often expressed that laevulose is utilised to a considerable degree by people suffering from glycosuria. The results recorded show that laevulose is utilised to a less degree than either cane sugar or starch.

56 g. of laevulose dissolved in 224 cc. of water were taken at 8 p.m. The comparative results depicted in Fig. 3 show the effect on the sugar excretion of this ingested laevulose.

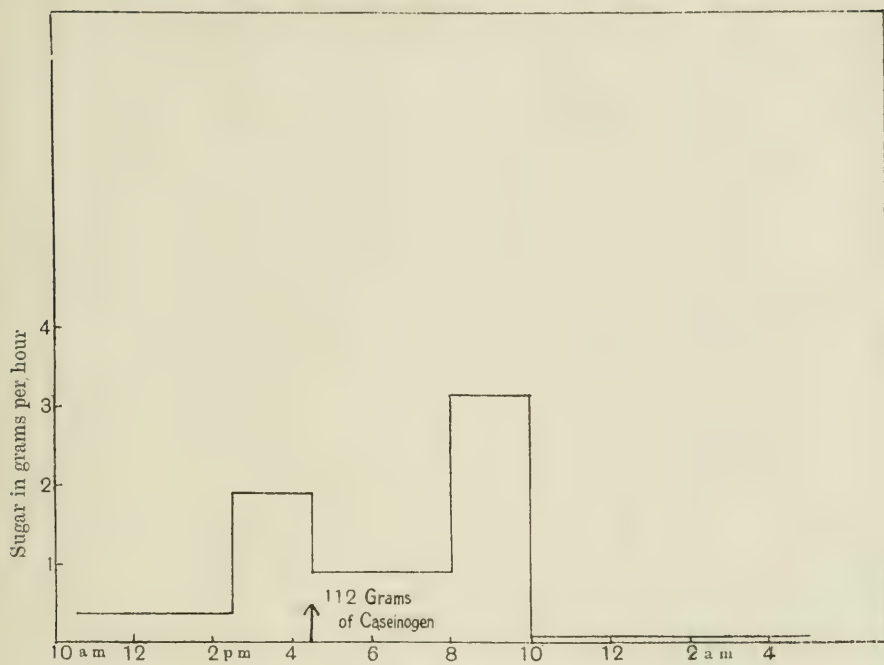


Fig. 2.



Fig. 3.

Time	Amount of urine	cc. KMnO_4 for 1 cc.	Total sugar	Sugar per hour	Urine per hour
	cc.		g.	g.	cc.
3 to 5 p.m.	140	7.7	4.04	2.02	70
8 "	56	9.2	1.93	0.64	19
8.30 "	168	9.2	5.85	11.7	336
10 "	280	15.1	16.1	10.7	187
1.30 a.m.	280	12.0	12.7	3.65	80
3 "	70	10.5	2.8	1.86	46
6 "	112	5.0	2.1	0.7	37

The curve shows that the ingested laevulose caused a very rapid rise in the amount of sugar excreted in the urine. Within 30 minutes the rate of sugar excretion increased from 0.64 g. to 11.7 g. per hour. The results also show that the greater part of the effect passed off in two hours since after that time the average rate of sugar excretion fell from 10.7 g. to 3.65 g. per hour. The figures allow an approximate calculation to be made of the amount of sugar excreted after eating 56 g. of laevulose. The average sugar excretion before eating the laevulose at 8 p.m. was 0.64 g. per hour. It again fell to this level at 3 a.m. It may therefore be concluded that the sugar excreted, due to the ingested laevulose, was confined to the urine formed between 8 p.m. and 3 a.m. Also it may be assumed that during this period 0.6 g. of sugar per hour was due to the metabolism of foodstuffs, other than laevulose, previously eaten. On these assumptions it may be calculated that 33 g. of sugar were excreted as the result of eating 56 g. of laevulose—or of the laevulose ingested 41 % was utilised whilst 59 % was excreted in the urine. Comparative colour tests showed that practically the whole of the reducing sugar excreted was dextrose. The urine passed at 8.30 p.m. and 10 p.m. gave a characteristic Selivanoff reaction. The colour produced under standard conditions when compared with the colour produced under similar conditions in normal urine to which known amounts of laevulose had been added indicated that only 0.75 g. of laevulose was excreted as such. Therefore the following conclusions may be summarised from these observations: (a) nearly 40 % of the ingested laevulose was excreted as dextrose, and (b) within two hours two-thirds of the excreted sugar had been so transformed and excreted.

(ii) *Cane sugar.* In order to determine the capacity of the man to utilise this form of carbohydrate, 112 g. of cane sugar were dissolved in 450 cc. of water. The syrup was taken at 7.15 p.m. The following figures show the amount of sugar excreted during the experimental period:

Time	Amount of urine	cc. KMnO_4 for 1 cc.	Total sugar	Sugar per hour	Urine per hour
	cc.		g.	g.	cc.
11.15 a.m. to					
4.30 p.m.	140	6.6	3.52	0.67	26.5
8 "	84	4.6	1.52	0.435	24.0
1 a.m.	392	17.4	25.8	5.2	78.0
4 "	140	21.3	11.4	3.8	47.0
7 "	56	4.0	0.85	0.28	8.0

These results, shown graphically in Fig. 4, illustrate in a clear manner the effect of eating 112 g. of cane sugar on the amount of sugar excreted in the urine. At 8 p.m., immediately after eating the cane sugar, the average amount of sugar per hour in the urine was 0.435 g.; up to 4 a.m. there was a large rise in the excretion, and after that time the average per hour fell to 0.28 g. It may be concluded that the sugar excreted at 1 a.m. and 4 a.m. was mainly derived from the ingested cane sugar. On this assumption, and allowing 0.3 g. of sugar per hour as derived from some source other than the cane sugar, it may be calculated that the ingestion of 112 g. of cane sugar resulted in the

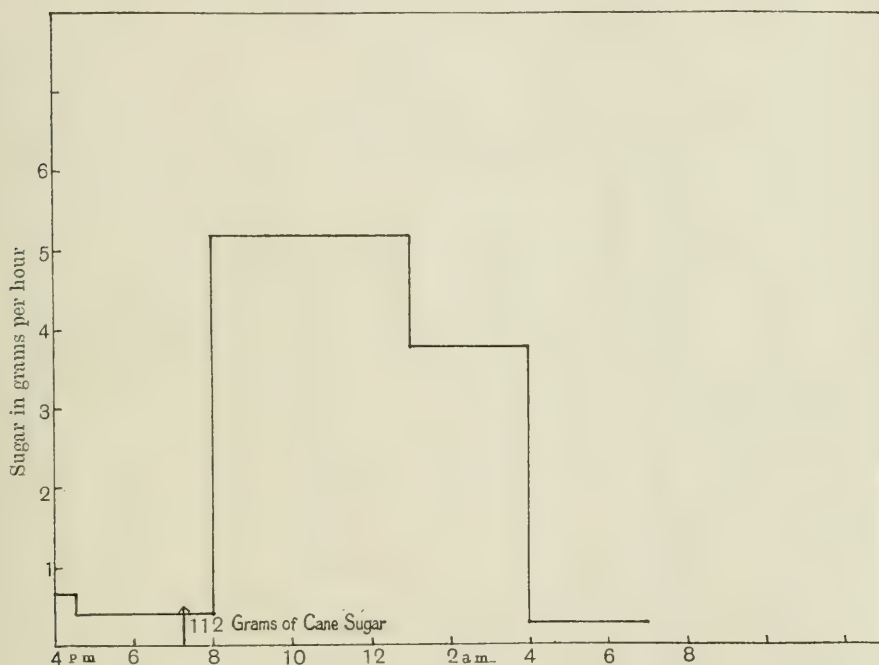


Fig. 4.

output of 36.5 g. of sugar in the urine. There was no cane sugar contained in the urine, since its reducing power was unchanged after hydrolysing with 1 % H_2SO_4 at 120° for 30 minutes. Also there was only a trace of laevulose in the urine—less than 0.1 g. Hence we may conclude that the 36.5 g. of reducing sugar contained in the urine consisted of dextrose only. Now 112 g. of cane sugar when hydrolysed yield 124 g. of invert sugar. Therefore after the absorption of 124 g. of invert sugar from the alimentary canal 87.5 g. were utilised by the tissues whilst 36.5 g. were excreted as dextrose. Despite the fact that the man excreted sugar not only on a carbohydrate diet but also on a protein diet, his tissues possessed a capacity to utilise carbohydrate derived from cane sugar to the extent of 71 %, only 29 % being excreted as dextrose in the urine.

(iii) *Oatmeal*. This foodstuff has been advocated by Van Noorden as a suitable diet for diabetics. The capacity of the man to utilise carbohydrate when presented in this form was therefore determined. On the first occasion the man ate 23 g. of oatmeal at midday. The following figures show the effect of this food on his excretion of sugar.

Time	Amount of urine	cc. KMnO_4 for 1 cc.	Total sugar	Sugar per hour	Urine per hour
8.30 a.m. to	cc.		g.	g.	cc.
10.30 a.m.	84	2.05	0.65	0.33	42
3 p.m.	84	1.05	0.34	0.07	18
4 "	252	2.4	2.28	2.28	252
10 "	168	5.5	3.5	0.87	42
2 a.m.	280	0.25	0.26	0.06	70

The results are shown graphically in Fig. 5. It may be seen from the diagram that the ingestion of 23 g. of oatmeal resulted in the excretion of 5.78 g. of dextrose. Now 23 g. of oatmeal contain 14.7 g. of starch, and this on hydrolysis yields 16.4 g. of dextrose. Therefore a diet containing carbohydrate equivalent to 16.4 g. of dextrose resulted in 10.6 g. being retained by the tissues and 5.8 g. being excreted in the urine. In other words 35 % of the available carbohydrate was excreted as dextrose whilst 65 % was utilised by the tissues of the body.

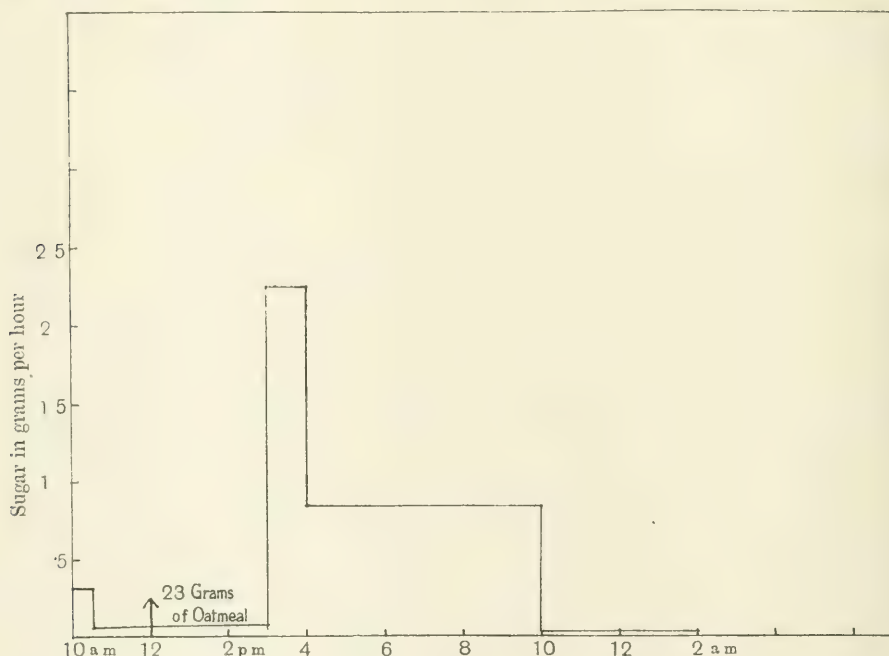


Fig. 5.

A second experiment was made in which 184 g. of oatmeal were given in four equal portions, *i.e.* 46 g. at 5 a.m., 8.30 a.m., 12.30 p.m. and 4.30 p.m.

The object of the experiment was to determine whether he could utilise carbohydrate to a greater or less extent when it was continuously supplied to the tissues of the body during the day, in contradistinction to the above experiment in which a small quantity of carbohydrate was given at one meal. The following results were obtained:

Time	Amount of urine cc.	cc. KMnO_4 for 1 cc.	Total sugar g.	Sugar per hour g.	Urine per hour cc.
1-5 a.m.	112	9.1	3.9	0.97	28
9.30 "	168	14.0	8.9	2.0	37
Noon	168	13.3	8.5	3.4	67
3 p.m.	168	13.0	8.25	2.75	56
5 "	84	12.4	3.95	1.97	42
10 "	252	18.8	17.9	3.6	50
2.15 a.m.	112	9.5	4.03	0.95	26

The results are plotted in Fig. 6. The graphic record shows that the sugar excretion due to the oatmeal eaten at the four meals during the day extended from 9.30 a.m. to 10 p.m. During this time the amount of dextrose excreted (allowing 1 g. of sugar per hour as derived from sources other than the oatmeal, *i.e.* the quantity excreted per hour before and after the experimental period) amounted to 35.5 g. The oatmeal eaten contained 118 g. of starch and this on hydrolysis would yield 130 g. of dextrose. Therefore of the total carbohydrate (calculated as dextrose) available during the day approximately 94.5 g. were metabolised by the tissues of the body and 35.5 g. were excreted in the urine; or in other words, 73 % was metabolised and 27 % excreted.

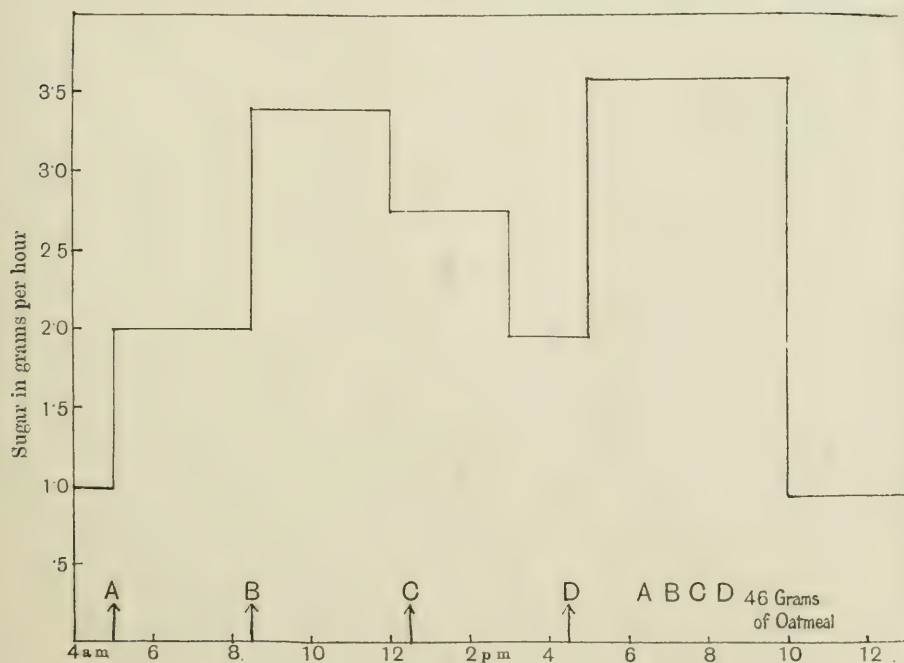


Fig. 6.

EXPERIMENTAL RESULTS.

The results detailed in the previous pages as to the relation of sugar excretion to diet in a man suffering from a moderate form of *diabetes mellitus* are summarised below:

(1) When first determined the daily output of sugar was about 250 g. contained in 4 litres of urine. Starvation freed the urine from sugar in 60 hours. Three weeks later the sugar excretion per diem was 120 g. contained in 3 litres of urine. A second period of starvation rendered the urine sugar-free in 24 hours.

(2) Whatever the carbohydrate eaten—starch, cane sugar, lactose or laevulose—dextrose only was excreted in the urine.

(3) Starch and cane sugar were utilised to the extent of 70 %; laevulose, on the other hand, was utilised only to the extent of 40 %.

(4) The capacity of the tissues to utilise sugar was not an absolute amount but was determined by the quantity of carbohydrate presented to them. Thus when 14.7 g. of starch were eaten 65 % was metabolised by the tissues and 35 % was excreted as dextrose. When 118 g. of starch were eaten 73 % was utilised by the tissues and 27 % excreted as dextrose. In the latter case nine times as much carbohydrate was metabolised as in the former case.

(5) Amino-acids, contained in caseinogen, available for conversion into dextrose, were utilised to a greater extent than starch. Thus only 6 g. of dextrose were excreted in the urine after eating 112 g. of caseinogen, *i.e.* 89 % of the amino-acids available for conversion into dextrose were metabolised.

(6) Within two hours of the ingestion of 56 g. of laevulose 30 g. of dextrose were excreted in the urine.

(7) The absorption of extractives of small energy value from the alimentary canal produced no increased excretion of sugar.

(8) Considerable quantities of alcohol were metabolised without a concomitant excretion of sugar in the urine.

The following additional facts have been observed in other cases of *diabetes mellitus*.

(9) In *diabetes mellitus* there is a marked hyperglycaemia. The amount of dextrose in the blood may exceed 0.4 %. During starvation the amount of sugar in the blood diminishes and reaches a normal level when the excretion of sugar in the urine ceases.

(10) The liver of a child whose death was due to *diabetes mellitus* possessed as great an action on carbohydrate and fat as that of a child suddenly killed by accident.

DISCUSSION OF RESULTS—THE ABNORMAL FACTOR IN *DIABETES MELLITUS*.

Various hypotheses have been advanced as to the causation of this disease. Among such hypotheses may be mentioned those which assign the abnormality to (a) the alimentary canal, (b) the liver, (c) the muscles. The experimental results given in the previous pages offer some evidence in favour or against these hypotheses.

(a) *The alimentary canal.* The hypothesis that the essential cause of diabetes is an alimentary toxæmia received strong support from the results obtained by starvation on the excretion of sugar. But the observations that some cases of diabetes continue to excrete sugar even after prolonged periods of starvation, and that the absorption of water, alcohol or extractives from the alimentary canal does not increase the excretion of sugar, militate against this hypothesis.

(b) *The liver.* In many fatal cases of diabetes the liver is found enlarged. This fact, together with the knowledge of the part played by the liver in carbohydrate metabolism, forms the basis of the hypothesis that in *diabetes mellitus* the liver is at fault. The assumption that hypertrophy is due to an attempt on the part of the organism to compensate for a diminished functional activity receives no support from a consideration of the ferment activity of the organ. Within two hours of the ingestion of 56 g. of laevulose 30 g. of dextrose were excreted in the urine—a chemical change which may be assumed with some degree of certainty to have occurred in the liver. Further, preparations made from the liver of a child who had died from diabetes were no less active on various forms of carbohydrate and fat than similar preparations made from the liver of a child accidentally killed. Apart from the question of ferment activity, however, it might be assumed that the essential failure in the chain of carbohydrate metabolism in *diabetes mellitus* is the inability of the liver to form compounds, analogous to phosphatides in fat metabolism, for the transport of dextrose to the muscles. This assumption is negatived by the fact that in diabetes the body can utilise to an equal degree carbohydrates presented to it in large or small quantities. It is clear that any synthetic deficiency would be more marked the greater the quantity of substance to be elaborated.

(c) *The muscles.* Cohnheim's [1903] hypothesis that there is a deficiency of pancreatic kinase required to activate the precursor of the glycolytic ferment in muscle—diabetes being due to a failure of the muscles to utilise dextrose, secondary to a disorder of the pancreas—was not supported by the experimental results of Patterson and Starling [1913] on the rate of utilisation of dextrose by the perfused hearts of depancreatized dogs. They demonstrated that pancreatic glycosuria in dogs does not result in the production of such a condition that cardiac muscle is unable to utilise dextrose presented to it in a perfusing fluid.

Again, there is no evidence that the muscles are unable to utilise dextrose in diabetes. Muscular weakness is never a prominent symptom except in advanced cases of the disease. Further, many observations have been made

showing that even with pronounced glycosuria no acetone or allied bodies may be present in the urine. Now an early sign of carbohydrate deficiency in the body is the excretion of β -hydroxybutyric acid and acetone. Therefore, in these cases, it is clear that the tissues are able to elaborate all the carbohydrate they require for their metabolic activities. In fact, if the quantity of dextrose present in the blood is such that the muscle cells can elaborate an amount of energy complex commensurate with their requirements, then the concomitant excretion of sugar by the kidneys does not involve any evidence of muscular weakness or the excretion of acetone bodies in the urine. The fundamental fact observed in the experiments recorded is that the capacity of the tissues to utilise carbohydrate is not an absolute amount but is determined by the quantity of carbohydrate presented to them. Now an established law of ferment action is that the quantity of substrate changed by a ferment is proportional to its concentration. We must therefore consider the carbohydrate ferments of the muscle cells as the weak link in the chain of carbohydrate metabolism. The following hypothesis is put forward to elucidate the phenomena observed.

The muscles in *diabetes mellitus* are able, to a limited extent only, to utilise dextrose in the concentration at which it exists in normal blood, the degree of limitation being proportionate to the severity of the disorder. There is therefore a call for a higher concentration demanding more sugar from the sources of supply. Thus the percentage of sugar accumulates in the blood until it reaches a level at which it is excreted by the kidneys. On this hypothesis the accumulation of sugar in the blood and the resultant glycosuria are secondary to changes in the synthetic activity of the muscle ferments. The muscles are able to utilise dextrose but, compared with normal muscle, they are unable to synthesise the inogen complex at a rate commensurate with their expenditure of energy from dextrose present in the blood at a concentration below that at which it is excreted by the kidneys. Hyperglycaemia in diabetes is a compensatory mechanism. The resultant glycosuria is a secondary effect depending on the fact that so far as the tissues of the body generally are concerned the activities of the kidneys are so regulated that dextrose present in the blood in a percentage greater than a certain amount is excreted in the urine.

This hypothesis demands as a corollary that the muscles are the active agents which mobilise the carbohydrate stores of the body. We propose to consider the problem, whether the active agent is a chemical hormone elaborated by the muscles or whether the central nervous system is involved in the metabolic chain, in a subsequent communication.

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IX. NOTE ON THE RÔLE OF THE ANTI-SCORBUTIC FACTOR IN NUTRITION.

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(Received February 24th, 1919.)

CONSIDERABLE interest is focussed at the present time upon the antiscorbutic substance found in many natural foodstuffs, and the relationship of this substance to scurvy in man and to experimental scurvy in guinea-pigs and monkeys has been the subject of many investigations during the past few years. These researches have demonstrated that if individuals of these species are fed upon a dietary deficient in the antiscorbutic substance they will sooner or later exhibit the classical symptoms of scurvy. Other species, however, appear to be able to thrive for long periods of time upon similar dietaries, without showing apparent symptoms of ill-health, much less definite pathological lesions. Such a species is the rat. The researches upon the growth problem have been carried out very largely with this animal as an experimental subject, and they have yielded results which indicate that the rat requires for a satisfactory completion of its life cycle a diet containing not only adequately adjusted supplies of protein, fat, carbohydrate and inorganic salts, but also a sufficient supply of two accessory factors, which have been provisionally termed "fat-soluble *A*," and "water-soluble *B*." The general opinion has up to the present held that if the diet is adequate in these respects the rat will show a normal rate of growth and a normal standard of nutrition throughout the usual span. This has led to the assumption that the rat is a species representative of a type showing no susceptibility to scurvy, since to all intents and purposes it has been possible to obtain a normal standard of nutrition throughout its life period, although the diet has been seriously, if not totally, deficient in the antiscorbutic factor or "water-soluble *C*." The only alternative to such an assumption, as was pointed out by McCollum and Pitz [1917] is to conclude that scurvy is in reality not a deficiency disease in the sense in which the term has been employed during the past ten years.

Since this alternative has been shown to be unsatisfactory, and since it is now established without any doubt that scurvy is indeed a typical deficiency disease [Harden and Zilva 1918, 1], we are faced with the need for an explanation of the ability of the rat to grow and maintain apparently excellent health in the absence of the antiscorbutic substance.

Quite recently Harden and Zilva [1918, 2] have brought forward experimental evidence that the antiscorbutic factor does play a beneficial rôle in the nutrition of the rat. They state that rats subsisting on a diet containing the antiscorbutic substance as well as the water-soluble and fat-soluble factors grow better than rats from the diet of which the antiscorbutic factor is absent.

This, they point out, indicates that rats are susceptible to an antiscorbutic deficiency although they do not develop definite lesions of the disease. Some time prior to the publication of this paper the same point was under investigation in this laboratory, because some rough experiments had indicated that recovery from the pathological condition induced by a deficiency of fat-soluble *A* was in certain cases more rapid when a diet containing butter fat plus orange juice was given than when the ration contained butter fat but no fruit juice.

Two standard dietaries were made up as follows:

	Ration 1	Ration 2
Purified caseinogen	20 parts	20 parts
„ starch	50 „	50 „
Butter fat	20 „	20 „
Salt mixture	5 „	5 „
Yeast extract	5 „	5 „
Orange juice	0 „	10 „
Water	40 „	30 „

Several healthy litters of young stock rats of approximately the same age were selected and each litter was halved. Two batches were then made up of the halves of the divided litters, so that half of each litter would receive each dietary. At the commencement of the experiment the animals were about six weeks old and were all of very similar body weight. These two batches were fed, the one on the orange juice diet and the other on what may be termed the scorbutic ration.

Both lots grew well, and after maturity was reached breeding was permitted, and the growth and development of the second generation were also watched.

Throughout the experiment, more satisfactory development was shown by the batches receiving the orange juice addition to their diet. The difference was not marked until the animals approached maturity, and was not discernible to the eye, being only apparent in a study of the body weight.

Table I gives the average weights of male rats upon the two diets. All the animals in each batch showed body weights very close to the average, so that there was no case of one or two low weights causing an unfair reduction of the value.

Table I. *Average Weights of Male Rats in grams.*

Days	Ration 1 (6 animals)	Ration 2 (6 animals)	Donaldson [1915]
0	62	64	—
30	124	122	125
60	161	183	184
90	193	213	223
120	208	234	244
150	220	248	258
180	233	256	268
210	241	263	274
240	245	278	280
270	249	287	296

A similar observation was made in the case of the females, but in this case it is difficult to illustrate the fact clearly in tabular manner owing to the disturbances in the weight increments caused by pregnancies. In these two batches there was just as little to choose between the appearance of the animals as was observed in the case of the males. After maturity was reached the breeding propensities of both batches were good, although on the whole a larger number of litters was obtained from the females receiving the orange juice diet.

The representatives of the second generation nourished upon the two rations were in every case of good appearance, but Table II demonstrates that those receiving the orange juice supplement were able to grow at the more rapid rate. All litters were reduced to a uniform size (4) immediately after birth, so as to equalise the labour of rearing the young animals imposed upon the females of the two sets.

Table II.

Days	Males			Females		
	Ration 1 average of 9	Ration 2 average of 11	Standard value of Donaldson	Ration 1 average of 10	Ration 2 average of 18	Standard value of Donaldson
0 (birth)	5.7 g.	6.0 g.	—	5.7 g.	6.0 g.	—
7	14.5 "	15.0 "	—	14.0 "	14.6 "	—
14	23.5 "	23.5 "	—	22.6 "	22.6 "	—
21	34.2 "	36.0 "	30.0 g.	30.2 "	32.0 "	28.0 g.
28	48.0 "	52.0 "	48.5 "	48.0 "	50.0 "	41.0 "
56	122.0 "	146.0 "	110.0 "	102.0 "	105.0 "	100.0 "
84	165.0 "	204.3 "	173.0 "	132.0 "	146.0 "	143.0 "
112	197.0 "	226.4 "	213.0 "	146.0 "	159.0 "	166.0 "

Although these figures represent the averages of somewhat small numbers of animals, they may be taken as indicating that the rat requires the anti-scorbutic factor in order to achieve a normal development, and that although the requirements of this species are of a very much smaller order than those exhibited by man, the monkey or the guinea-pig, they are sufficiently well-marked to dispel any idea that there exists a fundamental difference in the nutritive requirements of the two types of animal.

These results are in agreement with those published recently by Harden and Zilva [1918, 2].

It may therefore be accepted as experimentally proven that the dietary requirements of the higher animals include in addition to a satisfactorily balanced ration of protein, fats, carbohydrate and mineral salts, an adequate supply of three accessory food factors:

1. Fat-soluble *A*.
2. Water-soluble *B*, or antineuritic factor.
3. Water-soluble *C*, or antiscorbutic factor.

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X. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. I. OBSERVATIONS UPON ITS NATURE AND PROPERTIES¹.

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OTHER than numerous observations upon the distribution of fat-soluble *A* in foodstuffs curiously little attention has been devoted to that important substance. The present study was begun with the object of ascertaining its chemical nature.

Few definite statements regarding the properties of fat-soluble *A* are to be found in the literature. By many it has been considered to be a relatively thermostable substance, a view which has led to suggestions being advanced that it was a more or less clearly defined chemical unit such as a lipoid. McCollum and Davis [1914, 1] reported that the factor was present in an ether extract of boiled eggs, whilst Osborne and Mendel [1915] found the growth promoting properties of butter fat unaffected by treatment with steam for over two hours.

More recently, however, the thermostability of the accessory factor has been questioned by Steenbock, Boutwell and Kent [1918], who have demonstrated that exposure of butter fat to a temperature of 100° C. for four hours is sufficient to destroy most, if not all, of its growth stimulating powers.

Regarding the chemical nature of fat-soluble *A* practically no information has been derived from a study of the literature. McCollum and Davis [1914, 2] hydrolysed butter fat in a non-aqueous medium at room temperature and, without attempting any separation of the products of hydrolysis, believed they had obtained evidence that fat-soluble *A* had survived the process.

A very considerable amount of work has been carried out in this laboratory aiming at the isolation and identification of fat-soluble *A*. Much of the earlier work, however, was based on an assumption that the factor is relatively stable to temperatures below 120°.

This assumption was founded partly on the results obtained by previous workers which have already been referred to, and partly on the observation

¹ The author wishes to express his indebtedness to the Medical Research Committee for a grant which defrayed part of the expenses of this investigation.

that many oils, in the preparation of which drastic processes employing high temperatures have been used, were found to be rich sources of fat-soluble *A*. Examples of oils of this type are many fish oils [Drummond, 1918].

Few of the early experiments will be described because they have scarcely any interest in the light of the results yielded by the examination of the stability of the accessory factor *A*.

The thermostability of this factor was not doubted until an enquiry into the effects of hydrogenation upon the factor present in oils was undertaken in collaboration with Professor W. D. Halliburton, at the request of the Oil and Fats Committee of the Food Investigation Board.

This investigation yielded results which demonstrated beyond any doubt that fat-soluble *A*, in the form in which it occurs in natural animal fats, is much less stable to high temperatures than has been previously assumed. Such an observation necessitated a complete revision of all the previous experiments to isolate and identify the factor, in many of which high temperatures had been employed.

EXPERIMENTAL METHOD.

At an early stage it became apparent that some more or less approximately standardised method of testing substances for the presence of fat-soluble *A* was necessary, if any results of a comparable nature were to be obtained. The elaboration of such a method presented very great difficulties, as may well be imagined by those who have had experience in this field of research.

Although the method to be described is far from perfect, it has nevertheless yielded somewhat more useful results than would have been obtained by direct feeding tests of the usual type. The method is at present being used as a routine in this laboratory and should be useful for comparative studies, when improved by certain modifications which are now under consideration.

The animals used in trials of this nature are selected from our own carefully bred stock, and are, therefore, of a higher standard than those which form the average stock supplied by outside breeders.

The beneficial results of using none but home-bred stock are quickly apparent in this type of research and should be borne in mind by anyone who attempts to undertake it. Young healthy rats selected from the main stock and weighing about 50 g. are fed upon an artificial ration constituted as given below:

Purified caseinogen	20 parts
„ starch	50 „
Salt mixture	5 „
Yeast extract (source of water-soluble <i>B</i>)	5 „
Butter fat (source of fat-soluble <i>A</i>)	15 „
Filtered orange juice (source of water-soluble <i>C</i>)	5 „

The last constituent is added because it has been recently shown that rats show a more satisfactory growth and development when their ration contains

the antiscorbutic factor in addition to the other requisites. [Harden and Zilva, 1918; Drummond, 1919.]

Whilst nourished upon this ration the young rats should show a strictly normal rate of growth and maintain good health. Any which fail to do so in this preliminary period are regarded as unfit for experimental purposes and are rejected. The remainder, having given evidence of a normal power to grow, are removed from the complete ration when they have attained an average body weight of 70 to 80 g. and are given a dietary similar to the above but in which the butter fat has been replaced by an equivalent amount of a fat known to be deficient in fat-soluble *A*.

For this purpose considerable use has been made of hardened linseed oil, which contains no detectable traces of the accessory substance *A*. Occasionally when a very important experiment was undertaken this fat was further purified by one recrystallisation from alcohol.

Fed upon this diet deficient in fat-soluble *A* the experimental animals should quickly cease growing. Occasionally a very vigorous individual will continue to grow for some weeks after the deficiency has been introduced and such animals should not be employed in experiments of a comparative nature. The majority of the young animals show greatly retarded growth after a week upon the deficient dietary. When it is definitely established that growth is inhibited by the deficiency of fat-soluble *A*, the linseed oil is either wholly or partially replaced by the substance to be tested. The behaviour of the animal is then closely watched during a period of from four to six weeks. An absence of fat-soluble *A* in the added fraction is indicated by no growth on the part of the animal, followed by a decline in health accompanied by the characteristic eye-condition which has somewhat loosely been termed a xerophthalmia.

On the other hand, if the animal shows growth, the rate of this may be taken to be roughly proportional to the amount of fat-soluble *A* which has been supplied by the added fraction. Finally, in cases where little or no growth has been observed during the period when the unknown substance was tested, the rats are placed upon the original complete dietary in order to determine whether they still possess the power to grow when all their nutritive requirements are supplied.

It will naturally be realised that it is of great importance to determine the food intake throughout experiments of this type, although there is no need for other than approximate values, unless the trial is one of a crucial nature. The importance of working along some such roughly standardised lines as these is quickly impressed upon one during the course of an investigation of this nature, for it has been ascertained that the requirements of animals for the fat-soluble accessory substance become smaller as maturity is approached, so that the age of the experimental animals and their food intake must be taken into careful consideration.

EFFECT OF TEMPERATURE ON FAT-SOLUBLE *A*.

As has already been stated this study arose from an enquiry into the effect of hydrogenation on the fat-soluble factor present in oils and fats. Very large quantities of oils are now hardened by the catalytic method of reduction with gaseous hydrogen, the products obtained being utilised widely for the manufacture of edible fats, such as butter and lard substitutes. [Halliburton, Noël Paton, Drummond and others, 1919.] The ever-increasing consumption of preparations of this type might conceivably lead in time to a grave disturbance of the health of the people, should it be found that they are seriously deficient in the fat-soluble factor.

Hardened vegetable oils are known to be useless as sources of fat-soluble *A* [McCollum, Simmonds and Pitz, 1916, 1; Halliburton and Drummond, 1917], but no information was available regarding the influence of the process upon animal fats. As a convenient product for examination whale oil was chosen. This oil contains fat-soluble *A* [Drummond, 1918], is readily procurable in large batches of uniform composition, is representative of a type which is extensively used for hardening, and does not require to be separated from other substances before use, as is necessary when butter is used.

The specimen of whale oil employed in most of this investigation was a mixed batch of uncertain origin. Its quality was of the commercial grade 0-1, and it was a clear pale yellow oil containing a small amount of crystalline "stearin."

For this oil I am indebted to Mr D. H. Simpson, Manager of Ardol, Ltd, Selby, Yorkshire, to whom I must also express my appreciation of his kindness in preparing several specimens of treated oils.

The main supply of oil was tested and found to be a satisfactory source of fat-soluble *A* (Table I, experiment 6). 20 % of this oil was apparently as efficient as 8 % of butter fat (Table I, experiments 1-5).

Mr Simpson kindly arranged for a batch of this oil to be hardened in the plant at Selby. This was effected by exposure of the oil to gaseous hydrogen at a temperature of 250° for four hours in the presence of a metallic catalyst. The pressure in the hardening chamber is usually about atmospheric.

A sample of the hardened product was forwarded to me and was a clean white fat resembling mutton fat both in taste and smell. It has entirely lost all trace of the fishy smell possessed by the original oil. The hardening process had, however, also destroyed all power to act as a source of fat-soluble *A* (Table I, experiment 7). Young rats fed upon a diet containing 20 % of this fat as a sole possible source of fat-soluble *A* failed to grow and sooner or later developed the characteristic infection of the external eye.

The question then arose as to whether destruction of the active principle had been caused by the reduction of an unsaturated body or by the elevated temperature employed in the process.

Table I.

No. of experi- ment	Substance tested for fat-soluble A, and percentage in diet						Average weights in g. of three rats every seven days						Remarks
							0	7	14	21	28	35	
1	2 %	butter fat	90	96	106	109	116	119	Comparative tests with butter fat
2	4 %	"	92	102	111	115	118	121	
3	6 %	"	100	111	122	131	140	141	
4	8 %	"	85	100	119	134	136	149	
5	10 %	"	85	99	119	137	151	156	
6	Stock whale oil	20 %	85	108	124	131	141	147	Fat-soluble A present
7	20 %	hydrogenated whale oil	90	93	105	90	—	—	A completely destroyed
8	20 %	whale oil heated 250° for 4 hours	101	108	112	118	109	—	A destroyed
9	"	"	100°	"	98	102	103	115	106	—	"
10	"	"	75°	"	92	96	99	97	—	—	"
11	10 %	butter heated 100° for 1 hour	75	77	80	78	—	—	"
12	"	"	100° for 2 hours	95	100	106	109	107	100	"
13	"	"	100° for 4 "	101	112	112	108	—	—	"
14	"	"	75° for 4 "	76	87	101	111	116	117	Some destruction
15	"	"	50° for 4 "	81	94	106	119	130	132	Slight "
16	10 %	butter fat heated 37° for 21 days (rancid fat)	80	95	113	131	132	135	V. sl. destruction
17	20 %	whale oil exposed to air at 37° for 18 days	72	78	76	75	—	—	Destruction of A
18	20 %	whale oil exposed to air at room temperature for 18 days	70	86	92	100	104	112	Some destruction
19	20 %	whale oil heated to 37° in absence of air for 14 days	72	85	90	96	—	—	" "
20	20 %	whale oil kept at room temperature in contact with O	76	94	116	133	140	—	No destruction
21	Aqueous extract representing 20 %	whale oil	91	108	114	109	106	—	A probably not present
22	20 %	water-extracted whale oil	80	97	104	116	122	132	No destruction
23	Dilute alcohol extract representing 20 %	whale oil	75	83	95	83	—	—	A not present
24	20 %	acid-extracted whale oil	80	97	110	122	129	130	Very slight loss
25	Alcohol extract representing 20 %	whale oil	73	85	96	106	109	—	A probably present
26	20 %	alcohol-extracted whale oil	79	99	110	121	120	126	Very slight loss
27	Unsaponifiable matter representing 15 %	butter fat	96	102	100	92	—	—	A absent
28	15 %	fatty acids from butter fat	88	86	97	92	—	—	"
29	Unsaponifiable matter representing 25 %	whale oil	85	101	109	106	94	—	"
30	20 %	whale oil fatty acids	72	78	82	76	—	—	"
31	Unsaponifiable matter + fatty acids from	25 % whale oil	80	80	92	106	92	—	"
32	Glycerol water representing 25 %	whale oil	91	90	72	63	—	—	"
33	Unsaponifiable matter representing 25 %	whale oil. Special preparation	80	89	86	90	—	—	"
34	20 %	fatty acids from whale oil. Ditto	75	80	89	92	86	—	"
35	Glycerol water representing 20 %	whale oil	81	88	90	82	—	—	"
36	Unsaponifiable matter + fatty acids from	20 % whale oil	78	93	105	91	—	—	A present?
37	Unsaponifiable matter + fatty acids +	glycerol water from 20 % whale oil	80	87	93	89	—	—	A absent
38	1 %	cholesterol	90	88	85	80	—	—	A absent
39	1 %	lecithin (Merck)	98	99	100	82	—	—	"
40	0.5 %	sphingomyelin fraction	70	68	72	66	—	—	"
41	0.5 %	phrenosin	95	93	90	86	—	—	"
42	Crude carotene fraction approx. 0.003 %	pigment	108	114	123	135	—	—	Slight traces of A present
43	0.003 %	crystalline carotene	111	114	113	104	—	—	A absent

To test this point a sample of the oil was subjected at Selby to the conditions of hardening without the presence of hydrogen. This sample, after exposure to 250° for four hours, was inactive as a source of fat-soluble *A* (Table I, experiment 8).

As this result indicated that heat was the destructive agent the effect of lower temperatures was investigated. In view of the previous findings of the American workers it was somewhat surprising to find that exposure of the oil to temperatures of 75° or 100° for four hours was sufficient to render it valueless as a source of the accessory dietary unit *A* (Table I, experiments 9 and 10). Just about this stage of the investigation the paper by Steenbock, Boutwell and Kent [1918] reached this country, and in view of the results described therein it was decided to carry out some experiments on butter fat.

Their observations upon the destruction of butter fat at 100° were confirmed by the results of these experiments. Exposure of butter fat to 100° for periods of from one to four hours destroys its growth promoting power entirely, so far as can be determined by experiments on young rats (Table I, experiments 11-13).

The effect of lower temperatures was also investigated and it was ascertained that the nutritive value of butter fat may be appreciably lowered by four hours' exposure to temperatures ranging from 50°-75° (Table I, experiments 14-15).

Interest was immediately focussed upon the probable nature of the destruction, for obviously fat-soluble *A* is a substance of a much more unstable nature than had been previously believed.

The chemical changes which occur in oils and fats on heating at relatively low temperatures are as yet imperfectly understood. They are grossly represented by alterations in the acid value and the iodine value, which indicate changes of a hydrolytic and oxidative nature. It was therefore possible that inactivation of fat-soluble *A* was a result of oxidation or of increase in the proportion of free acid present.

Both these changes occur when fats undergo the alteration known as rancidity. A sample of butter was permitted to become markedly rancid by exposure to a temperature of 37° in contact with air for a period of slightly over three weeks. The acid value of this sample showed that rancidity was well advanced. Tested upon rats, this sample appeared to have lost very slightly its value as a source of fat-soluble *A* (Table I, experiment 16). This result at first led to the adoption of the view that oxidation was a factor in destruction but the following experiments with whale oil soon dispelled that idea.

Samples of whale oil were allowed to become oxidised by contact with air when spread out in thin layers in porcelain dishes. One sample was kept at room temperature whilst the other was maintained at 37° in an incubator. Both samples absorbed oxygen as shown by a drop in the iodine values (Table II) and the films of oxidised oil which formed on the surface were

frequently broken so as to expose the lower layers. Oxidation under these conditions was permitted to go on for eighteen days.

The feeding tests upon the animal gave surprising results. The sample oxidised at 37° was quite inactive as regards fat-soluble *A*, whilst the sample which had been kept at room temperature had lost but little of its activity (Table I, experiments 17 and 18). The iodine value of the two samples showed that oxidation had been more extensive at 37°, so that it was necessary to test whether this was responsible for the result.

A bottle was completely filled with oil, closely stoppered and sealed, after which it was maintained at 37° for fourteen days. This oil had undergone no changes of an oxidative nature as a result of this treatment as was shown by the iodine value (Table II). It had, however, very appreciably decreased in efficiency as regards fat-soluble *A* (Table I, experiment 19). A further sample of oil, given opportunity for oxidation at room temperature by prolonged shaking in the presence of gaseous oxygen, when tested was found to have lost little if any of the accompanying accessory substance (Table I, experiment 20). Apparently these experiments prove that oxidation plays no primarily important part in the destruction of fat-soluble *A* in animal oils.

This result confirms those obtained in the experiments described by Steenbock, Boutwell and Kent [1918] who found that butter fat was as rapidly inactivated by shaking for twelve hours at 40–60° with carbonated water as when atmospheric oxygen was present.

Table II.

Sample of oil						Value as source of fat-soluble <i>A</i>	Acid value	Iodine value
Original whale oil	+++	4.9	118.5
Whale oil heated 100° for 4 hours	—	9.5	116.2
“ “ 75° “	—	9.2	116.0
Whale oil exposed in thin layer to air at 37° for 18 days	—	6.5	97.7
Whale oil maintained at 37° in absence of oxygen for 2 weeks	+	4.95	118.7
Whale oil exposed in thin layer to air at ordinary temperature for 18 days	++	3.55	112.0
Whale oil shaken in contact with gaseous oxygen for 2 weeks	+++	4.82	114.0

It remains, however, to reconcile these findings with that described in an earlier part of this paper, where butter was found to have lost but little of its fat-soluble *A* after exposure to 37° in contact with air for over three weeks.

So far the only explanation of the different behaviour of the two oils is that butter fat is a much richer source of fat-soluble *A*. It is, therefore, possible for destruction to have occurred, and yet sufficient to have been left untouched to supply the requirements of the rats when their diet contained 10 % of the preparation. As a matter of fact the animals on which the rancid butter fat was tested did not show quite as satisfactory a rate of growth as those which received the untreated fat.

As all the experiments up to this point had been carried out on what may be termed a secondary source of fat-soluble *A*—that is an animal fat containing the accessory substance derived from plant sources—it appeared desirable for tests to be made of the thermostability of the factor in its primary environment.

Accordingly, a series of experiments was begun upon the factor as present in green leaves and seed embryos.

Neither of these investigations has yielded definite results and they are as yet incomplete. The experiments with cabbage gave evidence that drying may reduce the efficiency of leaves as a source of fat-soluble *A*, but no definite opinion can yet be given. The experiments with wheat embryo were a complete failure. Whenever this foodstuff was present to any considerable proportion in their diets the animals soon showed disturbances of health which quite upset the purpose of the experiments.

This point has not been investigated further as it leads too far away from the main enquiry. It is, however, interesting to note that McCollum, Simmonds and Pitz [1916, 2] have described the presence in wheat embryo of a substance distinctly toxic for rats.

A recent paper by Osborne and Mendel [1919] contains information that spinach and alfalfa are rich sources of fat-soluble *A*, even after the leaves have been dried at 50–60° in the air, but they give no comparative tests with the fresh vegetables.

Delf [1918], and Delf and Skelton [1918], report observations which lead them to believe that high temperature or the drying of cabbage leaves may effect a destruction of the fat-soluble accessory.

ATTEMPTS TO FRACTIONATE FATS CONTAINING FAT-SOLUBLE *A*.

Extraction of oils with water.

It was stated by McCollum, Simmonds and Steenbock [1917] that shaking melted butter fat with repeated changes of water removed the fat-soluble accessory substance from the fat. Many attempts to repeat this apparently simple method of separation were made in this laboratory. Butter fat, whale oil and cod liver oil were all treated as described but in no case was an active aqueous extract obtained. In the case of butter fat confirmation of their observation that the fat becomes inactivated by this treatment was obtained, but whale oil and cod liver oil appeared to lose little of their activity during the process (Table I, experiments 21 and 22).

One of the authors responsible for the original statement has now amplified it somewhat [Steenbock, Boutwell and Kent, 1918]. These investigators report that neither the washed butter fat nor the aqueous extract contains fat-soluble *A*. The factor had been destroyed during the process and their further experiments indicate that this is due to the temperature used during the extraction.

Extraction of whale oil with dilute acid.

At one time there was a suggestion that butter fat contained substances containing nitrogen and phosphorus which might be removed by extraction with dilute acids. Funk and Macallum [1914] were under the impression that a substance of the accessory factor type might be removed by this process.

Whale oil was extracted with three changes of 5 % hydrochloric acid for several hours at room temperature by repeated shaking. The acid extract after neutralisation with dilute sodium hydroxide solution was evaporated *in vacuo* at 30° to half its original volume. This fraction was totally inactive as regards the fat-soluble factor whilst the extracted oil, after being freed from traces of acid by extraction with water, was practically as active as before treatment (Table I, experiments 23 and 24).

Extraction with alcohol.

As there are definite grounds for believing that fat-soluble *A* is soluble under certain conditions in alcohol [Osborne and Mendel, 1915], a sample of whale oil was repeatedly shaken with several changes of cold absolute alcohol at room temperature.

The alcoholic extracts after removal of the alcohol *in vacuo* at 32° were found to possess a slight activity, but a larger proportion of the fat-soluble factor remained behind in the extracted oil (Table I, experiments 25 and 26).

A few attempts to modify the experiments carried out by Osborne and Mendel [1915] and thereby obtain an alcoholic extract of butter fat containing a high concentration of fat-soluble *A* failed. Destruction of the active principle apparently occurred owing to the exposure to the temperature of boiling alcohol, whilst the extracts when prepared appeared to lose their activity somewhat readily on storage. This confirms the later observations of Osborne and Mendel [1916].

ATTEMPTS TO SEPARATE FAT-SOLUBLE *A* FROM THE PRODUCTS OF THE
HYDROLYSIS OF FATS.

Prior to the appreciation of the thermolability of fat-soluble *A* much work along these lines was carried out. Butter fat, cod liver oil, whale oil and liver tissue, all of which are rich in fat-soluble *A*, were hydrolysed by hot alkalis and various fractions were prepared from the saponified products. No trace of any activity was found in any of these products.

As soon as the temperature destruction was known it was obvious that any further attempts along these lines must employ less drastic methods of hydrolysis.

McCullum and Davis [1914, 2] have already advanced evidence that the accessory factor accompanying butter fat can resist the conditions necessary for the hydrolysis of fats in a non-aqueous medium at ordinary temperature.

The first experiments along these lines were made with butter fat.

This fat was saponified by the method of Henriques [1895], a process that completely hydrolyses the ordinary type of fats in 24 hours at room temperature. After removal of the solvents *in vacuo* at 35°, the soaps were dissolved in distilled water at 35° and the unsaponifiable matter removed by extraction with ether. The fatty acids were then liberated by a very slight excess of mineral acid and removed by ether extraction. Both fractions were well washed with water and freed from ether by evaporation under reduced pressure at 37°. Animal feeding tests showed no activity on the part of either fraction (Table I, experiments 27 and 28). A similar experiment was made with the products isolated from cold saponified whale oil and similar results were obtained (Table I, experiments 29 and 30).

The possibility of fat-soluble *A* being a mixture of two or more substances suggested itself and tests were made with the united unsaponifiable and fatty acid fractions. No activity was observed (Table I, experiment 31). The glycerol fraction after the removal of ether and neutralisation was also inactive (Table I, experiment 32).

Although the possibility of oxidation during the processes being the cause of the destruction was not considered probable, in the light of the results detailed in the earlier work on whale oil, nevertheless a more careful experiment was carried out.

A sample of whale oil was hydrolysed by Henriques' method, and throughout all subsequent operations involved in the separation of the various fractions, great care was taken to exclude air. Distillations and operations of that kind were conducted in an atmosphere of carbon dioxide, while the fractions when prepared were stored in highly evacuated and darkened desiccators.

Particular care was taken in the selection of the animals employed in the testing of these particular fractions. No evidence of the presence of fat-soluble *A* in the unsaponifiable matter fraction was obtained (Table I, experiment 33).

Two of these animals whose diet contained 20 % of the fatty acids derived from whale oil showed practically no growth during a period of observation of five weeks and at the end of that time exhibited the external eye trouble. They speedily recovered health and resumed growing when given the original whale oil ration (Table I, experiment 34).

The third animal grew during the whole five weeks although the rate was sub-normal throughout. It did not at any time exhibit sore eyes. This animal is not included in the average.

As a doubtful result was thus obtained in a very important experiment a further test was made in which two young rats whose growth had been stunted and in which eye disease had been produced by a deficiency of fat-soluble *A* were given the ration containing 20 % of the whale oil fatty acids. No resumption of growth has yet been observed during fourteen days and the eye condition is becoming progressively worse.

It is, therefore, concluded that there is no evidence which identifies fat-soluble *A* with any of the fatty acids occurring in whale oil, and the rat which was observed to grow on that fraction must have been one of those individuals which are frequently encountered and which exhibit a power to grow for long periods of time although receiving a diet seriously deficient in fat-soluble *A*.

When a diet containing both the unsaponifiable matter fraction and the fatty acid fraction was tested a slight initial growth was observed in some cases. This was not maintained, however, during the following weeks of observation (Table I, experiment 36). Likewise, no positive results were obtained when the glycerol fraction was tested (experiment 35) or when all three fractions were combined (experiment 37).

It must be remarked that in all these feeding tests the food consumption was determined, even if only approximately. In every case except where the dietaries contained the glycerol fractions derived from the hydrolysed oils the food consumption was satisfactory. This excludes any complications arising from a failure of the animals to partake of sufficient food to ensure an adequate intake of the substance being tested.

DISCUSSION.

Speculation as to the nature of "fat-soluble *A*" as it occurs in oils and fats is perhaps unwise until this investigation has advanced a few stages further.

It appears doubtful whether the substance known as fat-soluble *A*, in the form in which it occurs in animal oils, is a clearly defined chemical substance of comparatively simple constitution. Long before the discovery of the thermolability of the factor made such tests valueless, many attempts were made in this laboratory to identify fat-soluble *A* with certain known constituents of fats.

Unsuccessful attempts were made to replace the fat-soluble accessory factor in the diet by a number of the commoner fatty acids, glycerol, cholesterol, lecithin, sphingosin, phrenosin, kephalin and the lipochrome carotene (Table I, experiments 38-43).

The discovery of the destruction at comparatively low temperatures at once narrowed the field of possible identity very greatly.

Further, when it no longer appeared probable that oxidation was responsible for the destruction, it was possible to rule out many substances such as the unsaturated acids and the lecithins.

It is of interest to refer here to the question of the therapeutic value of cod liver oil. For many years this foodstuff has been widely employed in the treatment of diseases of malnutrition, and there have been numerous speculations as to why this particular oil should be of value in such cases.

Small traces of iodine and other substances are frequently found in cod liver oil, and by some these impurities were considered to be responsible for the high nutritive value of the oil. It has been shown, however, that this view

is incorrect, and that oils which are free from impurities containing phosphorus, iodine and sulphur may give as good results in the treatment of wasting diseases as those which are less pure [Williams, 1912, 1].

Osborne and Mendel [1914], after having discovered the beneficial influence of cod liver oil on growth, remarked that it is perhaps more than a coincidence that the oil has so long enjoyed a reputation for nutritive virtues which can scarcely be attributed to its fat content *per se*. The existence of an accessory substance in certain fats was at that time only just being suspected and it is interesting in the light of present knowledge to quote the final paragraph of their paper. "Perhaps experiences such as have been reported in this paper will pave the way for a clearer understanding of the physiological potency of natural products like butter, egg-yolk and cod liver oil, which have long enjoyed a popular, yet inexplicable, reputation for unique nutritive potency."

Williams [1912, 1, 2] has suggested that in the case of cod liver oil it is owing to the presence of peculiar unsaturated fatty acids. The work described in this paper does not tend to identify the fat-soluble *A* factor with any of the fatty acids present in the oils in which it is found, and the theory of Williams would appear to lack support.

From the results obtained during this study one is led to conclude that the chief agent in the inactivation of fat-soluble *A* is temperature. It must, however, be borne in mind that all these speculations refer solely to the factor as present in secondary sources, such as the animal oils. It is quite possible that the factor occurs in a different form in its primary environment in the plant tissues; in fact McCollum, Simmonds and Pitz [1916, 1, 2] have already intimated that the factor may be present in the embryos of seeds in combination with some cell constituents such as protein.

After much careful consideration of the experiments which have been described it is felt that there is justification for advancing the suggestion that the accessory growth promoting factor, provisionally termed "fat-soluble *A*," is not a clearly defined chemical substance but rather that it is a labile substance perhaps possessing characteristics resembling those of an enzyme.

Although this hypothesis is advanced with some hesitation it is the only one which has been found to fit in with the majority of the experimental results.

Attempts have been made in collaboration with Dr O. Rosenheim to determine whether the factor can pass through rubber membranes, but unfortunately the experiments have so far failed. At present it is believed that toxic substances are dissolved from the rubber dialysis membranes and pass into the solutions of the oil rendering it impossible to carry out successful feeding experiments with the final products. When a more satisfactory technique has been evolved for the purpose of this experiment it is hoped to gain information as to the class of substance to which fat-soluble *A* belongs.

Much further work will be necessary before a definite conclusion upon the important question of the identity of fat-soluble *A* can be arrived at, but in

the meantime it may be useful to test the possibility of it being an ill-defined and labile substance such as an enzyme.

It is surely legitimate to conceive the animal organism being as dependent upon the plant kingdom for a supply of an essential complex of that nature as it is for the supply of many indispensable substances of more clearly defined constitution.

I am indebted to Professor W. D. Halliburton for his interest and advice during the course of these experiments and to Dr O. Rosenheim for many valuable suggestions and for purified specimens of certain lipoids and carotene.

SUMMARY.

1. The fat-soluble accessory food factor *A* present in certain oils, as for example butter fat and whale oil, is readily destroyed by short exposure (one hour) to a temperature of 100°.

2. Destruction occurs, but is less rapid, when the exposure is to temperatures ranging from 50° to 100°.

3. Exposure of the oil to a temperature of 37° for several weeks may cause destruction of the accessory factor.

4. Destruction is apparently not a result of oxidation or hydrolysis.

5. Fat-soluble *A* is not extracted from oils by water or dilute acid.

6. Fat-soluble *A* is soluble in alcohol, and may be removed in small quantity from oils by cold extraction with that solvent.

7. Hydrolysis of oils in a non-aqueous medium at room temperature causes disappearance of fat-soluble *A*, as the factor is not found present in any of the fractions derived from the original oil.

8. So far, there is no evidence which suggests that fat-soluble *A* is not a single substance.

9. Fat-soluble *A* has not been identified with any of the recognised components of fats, such as glycerol, saturated or unsaturated fatty acids, cholesterol, lecithin, phosphatides or the lipochromes.

10. In view of the low temperatures at which destruction may occur it is suggested that fat-soluble *A* may be a labile substance of ill-defined constitution.

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XI. RESEARCHES ON THE FAT-SOLUBLE ACCESSORY SUBSTANCE. II. OBSERVATIONS ON ITS RÔLE IN NUTRITION AND INFLUENCE ON FAT METABOLISM.

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IS FAT-SOLUBLE *A* NECESSARY FOR THE ADULT?

FOR some years past it has been accepted that the fat-soluble accessory factor plays an important part in the nutrition and growth of young animals, but information regarding the requirements after maturity has been reached is scanty. A powerful stimulus to research along these lines was provided by the necessity for a drastic reduction of the fat-ration imposed during the war by the shortage of edible fats, and it at once became a matter of some urgency to determine whether fat-soluble *A* is only required during the period of growth or whether it is equally necessary for the maintenance of health in the adult.

In one particular case it has been experimentally proved that the adult animal requires a regular supply of accessory factors, and that is the case of the pregnant or lactating female [McCollum, Simmonds and Pitz, 1916, 1; Drummond, 1918, 1]. But the demand for the factors in this case might be accounted for by the requirements of the growing young, and might have no reference to the direct nutrition of the mother's body.

During the course of a large number of experiments carried out in this laboratory during the last few years it has been observed that as young rats approach maturity their requirements for fat-soluble *A* become markedly less. A rat which is nearly full grown can successfully complete its development upon an allowance of fat-soluble *A* which would be insufficient to permit any growth whatever in a much younger animal.

It was therefore possible that fat-soluble *A* is essentially a "growth" factor and might be dispensed with by the adult. Such was not, however, found to be the case.

A number of young full grown rats both male and female were fed upon a dietary deficient in fat-soluble *A* composed as follows:

Purified caseinogen	20 parts
„ starch	50 „
Salt mixture	5 „
Yeast extract	5 „
Hardened linseed oil	20 „

By experiments on young rats it has been ascertained that this ration contains no detectable traces of fat-soluble *A*.

Many of the adult animals nourished upon this dietary were able to live in apparently good health for considerable periods of time, but sooner or later they all showed a decline in body weight accompanied by an obvious decreased resistance to disease.

Of fourteen control male rats which received an adequate dietary con-

Table I. *Weights of eight adult male Rats maintained on Diet deficient in Fat-soluble A.*

No. of rat	Weight in g. every seven days															
	0	7	14	21	28	35	42	49	56	63	70	77	84	91	98	105
1	255	260	260	260	260	280	280	275	275	270	255	240	270	260	280	280
2	220	210	220	216	223	235	232	225	228	224	223	219	205	201	214	233
3	290	292	300	310	310	310	315	320	330	310	310	300	280	280	270	290
4	297	305	300	309	315	300	300	310	315	320	310	300	290	285	300	300
5	299	300	295	300	300	310	295	305	295	285	250	255	240	240	240	218
6	306	310	310	320	310	305	300	297	300	296	290	299	296	282	290	292
7	350	350	350	350	347	356	350	346	346	340	339	345	335	330	329	326
8	268	270	282	296	290	295	290	286	288	290	290	290	296	300	294	290

No. of rat	Weight in g. every seven days										External eye disease	Cause of death
	112	119	126	133	140	147	154	161	168	175		
1	280	270	250	255	260	266	250	245	235	230	Slight 140th day Severe 150th day	Died 182nd day, bronchopneu- monia
2	227	225	233	225	220	197	165	140	—	—	Severe 143rd day	Died 162nd day, cause not clear
3	305	313	320	320	315	310	295	295	300	285	Slight 160th day	—
4	300	305	300	290	295	290	285	280	275	250	Slight 150th day Severe 160th day	—
5	—	—	—	—	—	—	—	—	—	—	—	Died 107th day, lung disease
6	276	260	—	—	—	—	—	—	—	—	Slight 90th day Severe 102nd day	Died 119th day, bronchopneu- monia
7	325	316	305	300	297	290	287	262	—	—	Slight 122nd day Severe 129th day	Died 163rd day, cause not ascer- tained
8	290	280	286	280	270	260	262	260	250	242	Slight 150th day Severe 155th day	Dying with lung disease at end of experiment

taining butter fat instead of hardened linseed oil twelve were alive and in excellent health at the end of the thirty weeks period of observation. One died from bronchopneumonia while another succumbed to sepsis consequent on injuries received in fighting.

Eight adult male rats were fed upon the deficient ration and their subsequent behaviour is recorded in Table I. Only three survived the experimental period, and they were all in a very poor nutritive condition. Many deaths were due to lung diseases, whilst practically every animal showed the characteristic external eye disease at some time during the experiment.

A number of young adult females were also kept under observation and fed upon the fat-soluble deficient dietary. Breeding with the males upon the same diet was permitted. The females themselves behaved very much as did the males described in Table I. After a period during which they maintained more or less good health they began to lose weight and to present an impoverished condition. Many deaths from infective diseases occurred and cases of external eye disease were frequent.

A number of litters were born, particularly during the first few weeks of the experiment, when the bad effects of the diet had scarcely begun to be felt. These early litters were usually of normal weight when delivered, but failed to grow at a normal rate owing to the deficient nutritive value of the mothers' milk [compare McCollum, Simmonds and Pitz, 1916, 1; Drummond, 1918, 1].

The majority were reared until able to look after themselves, but they all appeared puny and very quickly contracted infections, particularly the eye disease, and the mortality amongst them was very high.

A few litters were born after the females had been on the deficient ration for many weeks, but most of the young which comprised these litters were devoured by the mothers shortly after delivery, and any which escaped that fate seldom survived for more than a day or two. They were in nearly every case undersized when born.

It may therefore be taken as proved that the adult animal organism requires a regular supply of fat-soluble *A*. This daily requirement is of a much smaller order than that of the young growing animal, but nevertheless an important factor in the maintenance of health. It appears probable that the resistance to diseases of bacterial origin is seriously impaired by a failure of the animal to obtain a sufficient supply of the fat-soluble factor. There is therefore every reason that great care should be taken to ensure that dietaries of adults contain an adequate supply of foodstuffs in which fat-soluble *A* is present.

IS FAT-SOLUBLE *A* CONCERNED WITH THE METABOLISM OF FATS?

Up to the present we are in complete ignorance of the function of fat-soluble *A* in the body. The modern theories regard fat-soluble *A* as synthesised by the plant, in the tissues of which it may be present in a loosely combined

form which is insoluble in fat solvents. It is conceived that in the alimentary tract of animals this complex is broken down and the factor *A*, now in a form soluble in fats and fat solvents, forthwith accompanies them during absorption [McCollum, Simmonds and Pitz, 1916, 2].

Its fate after absorption into the animal body is as yet unknown. Its presence in certain depôt fats, such as beef fat, suggests that it may be in part stored in company with the reserve fat.

In view of the possible connection between fat-soluble *A* and some phase of the metabolism of fats a few experiments have been made which will be described in this paper.

It was first decided to test whether the animal organism is able to survive as well on a diet free from both fat and the fat-soluble factor as when the latter factor alone is absent.

Osborne and Mendel [1912] have reported the ability of young rats to show considerable development upon diets containing, at the most, very small traces of fat. It is probable however that the fat-soluble factor was present as an impurity in certain of the food components.

Three batches of six nearly full grown male rats of approximately the same age and weight were selected from the stock. Each lot was then given one of the three following dietaries.

	A	B	C
Purified caseinogen	20 parts	20 parts	20 parts
„ starch	70 „	50 „	50 „
Salt mixture	5 „	5 „	5 „
Yeast extract	5 „	5 „	5 „
Hardened vegetable oil	—	20 „	—
Butter fat	—	—	20 „

All three batches were kept under the closest observation for a period of three months.

Lot C nourished upon the butter fat ration showed throughout the whole period a normal standard of nutrition. At the conclusion of the experiment they were killed and subjected to post mortem examination. With the exception of one rat which showed a small worm encysted in the liver, no abnormalities were noticed in any case.

Lot B received a diet which contained an adequate supply of fat, but no detectable traces of fat-soluble *A*. These animals maintained their weight well and showed no apparent symptoms of ill-health until towards the end of the second month when they one by one began to develop the characteristic condition of sore eyes.

Two deaths occurred in the early part of the third month from bronchopneumonia and at the conclusion of the experiment the surviving animals were losing weight and all suffering from the external eye disease.

These survivors were killed and carefully examined. In every case there was a large amount of body fat present and there was no indication of in-

anition. The organs all presented a healthy appearance as far as the eye could detect. The histological examination has not been completed.

The body temperature of the rats in this group remained normal, except in the two cases which contracted an acute infection of the lung. This observation has been confirmed by the records obtained from a large number of rats fed upon diets deficient in fat-soluble *A*. There is therefore no indication that lack of fat-soluble *A* brings about a disturbance of the heat regulating mechanism, as does a deficiency of water-soluble *B* [Drummond, 1918, 2; Dutcher, 1918].

The batch of animals fed upon ration A showed very similar behaviour to those nourished upon ration B. Their food consumption was throughout higher owing to the fact that the calorific value of their diet was lower than that of diet B. They all maintained a good appearance until towards the end of the second month when sore eyes began to appear. Body weights then showed declines and there was one death from lung trouble. The remaining five at the end of the third month had all lost weight and exhibited more or less severe external eye trouble.

Post mortem examination of these animals failed to reveal any gross lesion, except in the lungs which were congested in some cases. The amount of body fat was apparently normal. The histological examination of the organs has not yet been completed.

These experiments indicate that the appearance of the symptoms associated with a deficiency of fat-soluble *A* is not postponed by withholding fat from the diet.

It has recently been shown that rats may live in perfect health for several months upon a diet containing relatively large amounts of free fatty acids [Halliburton, Paton, Drummond and others, 1919]. The classical researches of Munk [1880, 1884] showed that free fatty acids are absorbed from the intestinal tract and combined with glycerol derived from the body to form neutral fat.

The possibility of fat-soluble *A* playing a rôle in the resynthesis of fats suggested itself and an attempt to obtain information on this point was made. Two batches of rats were fed upon the following rations:

				Diet B	Diet D
Purified caseinogen	20 parts	20 parts
„ starch	50 „	50 „
Salt mixture	5 „	5 „
Yeast extract	5 „	5 „
Hardened vegetable oil	20 „	—
Fatty acids derived from hardened oil				—	15 „

The period of observation was as in the last experiment three months. The batch receiving fat but no fat-soluble *A* showed a behaviour closely similar to that described in the previous case. At about the end of two months symptoms of ill-health and external eye disease appeared and the animals gradually declined.

The batch which received the fatty acid ration (diet D) ate their diet with avidity throughout the experiment. After the lapse of some seven weeks ill-health began to appear accompanied as in the other cases by the characteristic condition of sore eyes.

Analyses of the faeces made on several occasions during the three months indicated that the absorption of the fatty acids was excellent, the highest value being 92 % and the lowest 86 %. At the expiration of the three months the surviving animals were killed—two had died during this period, one from bronchopneumonia and the other as a result of an accident.

Post mortem examination showed no obvious abnormality. Body fat was plentiful and all organs appeared normal as far as the naked eye could detect.

These animals had, therefore, been capable of absorbing large amounts of free fatty acids and of effecting the synthesis of these into neutral fats over a considerable period of time in the absence of any detectable traces of fat-soluble *A* in the diet. It is therefore justifiable to assume that this factor plays no important part in the synthesis of fatty acids into fats following absorption, for the power to absorb fatty acids and effect fat synthesis is apparently retained after the animal has passed the period during which it was able to maintain health in spite of the deficiency, and during which it is surmised that it may have been utilising reserve supplies of the essential factor.

The appearance of the characteristic symptoms associated with a deficiency of fat-soluble *A* in approximately the same time whether the ration is deficient in fat, contains an ample supply of fat or merely a supply of fatty acids, is an interesting observation. It has been confirmed in two out of the three cases in an experiment with young rats.

Two batches of twelve young rats were fed upon diets A and B; that is one lot received fat and the other none, whilst neither obtained fat-soluble *A*. The two groups were as far as possible comparable, and the following data were obtained as a result of close observation.

	12 young rats fed on diet A con- taining neither fat nor fat- soluble <i>A</i>	12 young rats fed on diet B con- taining fat but no fat-soluble <i>A</i>
Average weight at commencement of experiment	80.2	82.8
Average weight at 10 days	89.0	92.1
Average weight at 20 days	93.4	97.1
Average weight at 30 days	86.4	88.2
Average weight at 40 days	80.1	80.6
First appearance of case of sore eyes	28th day	26th day
Number of cases of sore eyes at 40th day ...	6	9

These observations appear to have an indirect bearing upon the question of the dispensability of fat in the diet of the animal organism. If an animal is deprived of fat upon an artificial ration of the type employed in this work it eventually succumbs to certain disturbances of nutrition which it is believed result from a deficiency of fat-soluble *A*. If it receives a pure fat it succumbs

in an exactly similar manner and after an interval of the same order. It therefore appears that no decision upon the question of the dispensability of fat can be arrived at until the fat-soluble factor has been obtained in a form free from fat. Then and then only can the nutritive effect of feeding diets deficient solely in fat be studied. But the onset of the symptoms alluded to above after the same interval, no matter whether the diet contains fat or not, does suggest that fat itself is a dispensable component of food.

The experiments recorded by Osborne and Mendel [1912] are of interest at this juncture. The diets they employed were practically fat-free, but as the components had not been extracted with fat solvents it is very probable that traces of the fat-soluble factor were present. Considerable growth over long periods was shown by young rats fed upon such rations.

INFLUENCE OF A DEFICIENCY OF FAT-SOLUBLE *A* ON FAT METABOLISM.

Two pairs of healthy rats weighing about 150 g. each were selected from stock and one pair was placed upon rations B and C respectively.

The pair which received ration C thrived for the whole three months during which they were under observation and were in excellent health at the close of the experiment. During this time their absorption of fat was studied for five periods of seven days. As will be seen from Table II, the absorption of fat was excellent in all three periods, averaging 97.3 %.

Table II.

Diet	Period of experiment :	Condition of the animals	Total consumption of dry food in g.	% fat in food	Total intake of fat in g.	Total weight of dry faeces in g.	% total fat in dry faeces	Total excretion of fat in faeces in g.	% utilisation	M. P. of fat in diet
C	1st week	Excellent	122	16.7	20.4	8.14	6.18	0.50	97.5	30.2°
C	3rd "	"	134	16.3	21.8	10.06	6.10	0.62	97.2	
C	6th "	"	141	16.3	23.0	6.02	13.30	0.80	96.5	
C	9th "	"	136	15.9	21.6	9.20	5.99	0.55	97.5	
C	12th "	"	142	16.1	22.8	9.42	5.72	0.54	97.6	
Average									97.3	
B	1st "	Excellent	126	15.2	19.2	9.09	14.2	1.29	93.4	38.5°
B	3rd "	Good	135	15.8	21.3	8.02	15.5	1.24	94.2	
B	6th "	"	132	16.4	21.6	9.11	14.5	1.32	89.2	
B	9th "	Ruffled appearance, one rat shows slight sore eyes	136	16.0	21.8	10.10	25.7	2.69	87.6	
B	12th "	Both losing weight and eyes very bad	126	15.7	19.8	7.12	30.4	2.16	89.1	
Average									90.7	

The two animals receiving diet B, which contained a fat known to be deficient in fat-soluble *A*, were studied in a similar manner. They showed good health for about six weeks but during the latter half of the period of observation they were failing and both contracted sore eyes during the third month.

Fat absorption was, however, good in all five periods, even in the final one when the physical condition of the animals showed that they suffered from a serious deficiency of fat-soluble *A*.

The coefficient of absorption shows a slight decrease in the later periods, but it is thought that this may represent a slightly lower efficiency of the digestive system consequent on the failing health. The reduction of the percentage absorbed is too small to be interpreted as evidence that fat-soluble *A* is directly concerned in fat absorption.

Estimations of fat in the faeces were carried out by the wet extraction method described by Saxon [1914].

SUMMARY.

1. Fat-soluble *A* is necessary for maintenance of health in the adult rat.
2. Further confirmation of the importance of supplying the female with an ample supply of fat-soluble *A* during pregnancy and lactation is given.
3. A deficiency of fat-soluble *A* in the diet of adult rats apparently brings about a serious lowering of the resistance against diseases of bacterial origin.
4. No obvious characteristic pathological lesion has been detected in adult rats which have been deprived of fat-soluble *A* for long periods. Such animals usually show normal reserves of body fat.
5. The symptoms associated with a deficiency of fat-soluble *A* appear in comparable groups of rats after the same period of time whether the diet contains neutral fat or not.
6. Rats are able to absorb large amounts of fatty acids, and presumably synthesise these into fats, in the absence of fat-soluble *A* in the diet.
7. A deficiency of fat-soluble *A* in the diet does not exert any direct influence on the absorption of fat.
8. Indirect evidence deduced from these experiments with rats suggests that pure neutral fats may be dispensable components of the diet.

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XII. NOTE ON XEROPHTHALMIA IN RATS.

BY ELSIE CHARLOTTE BULLEY.

(Received March 3rd, 1919.)

AMONG the diseases which have been attributed to a deficiency in the fat-soluble *A* accessory substance is a condition of the eyes now described as xerophthalmia. McCollum, Simmonds and Pitz [1918] indeed speak of this relation in very definite terms. "Xerophthalmia and polyneuritis are abundantly demonstrated to have their origin in the lack of a sufficient amount of the fat-soluble *A* and water-soluble *B* respectively in the diet." That eye symptoms in rats may occur as the result of deficiencies in the diet seems to be common experience. It is very important, however, to distinguish between symptoms due directly to a specific dietetic error and any that may be due to infection, encouraged, may be, by diminished resistance due to the diet.

My own experience seems to point to the fact that so far as xerophthalmia is concerned the latter relation is the true one. In considering this question I have taken absence of normal growth as evidence of a deficient ration.

It is desirable at the outset to view the origin and growth of the idea that xerophthalmia is due to the absence of some essential nutritive factor and later of the view that fat-soluble *A* is the pivot on which the matter turns.

Osborne and Mendel [1914, 1] in writing of their protein-free milk state that the addition of butter fat to this substance avoids failure in growth and "an infectious eye disease." They repeat this statement [1914, 2, 1915] when, dealing with the question of separating the essential fat factor, mention is again made of the fact that animals fed with an inefficient fat fraction developed purulent eyes. McCollum and Simmonds [1917] cite eye disease as a possible deficiency disease due to absence of the fat factor, and the writers quote cases of children developing it on fat-deficient diets. As previously mentioned [1918] they definitely state that xerophthalmia is brought about by absence of the fat factor. Osborne and Mendel [1918] make a further allusion to the condition stating that on a fat deficient diet the symptoms occurred, and were cured by the addition of liver oil to the food. In a recent paper by Harden and Zilva [1918] eye symptoms are again taken as indicative of the absence of fat-soluble *A*.

In January, 1913, a series of experiments was carried out in this laboratory solely with a view to clearing up this matter of eye disease, which had made its appearance amongst animals then being fed by Professor Hopkins on synthetic diets in which the mineral supply was Osborne and Mendel's artificial

salt mixture. The results of these experiments have never been published, and it is with Professor Hopkins' permission that I allude to them here. He found that animals fed on extracted starch, extracted caseinogen, sugar, salts, and lard developed an ulcerative condition of the cornea with conjunctivitis within 14 days from the commencement of the experiment, identical with that present in animals fed, in previous experiments, on a similar artificial diet, though these earlier rats had been fed with a different end in view. In the more extreme cases there was complete dissolution of the eyeball with rupture of the cornea. Many of the animals died, but one or two survived and on being given 3 cc. milk as an addendum to the diet, the eye symptoms cleared up. This led to the assumption that the disease was due to some deficiency in the diet which the 3 cc. of milk supplied. In 1915 Dr Ginsaburo Totani, at the suggestion of Professor Hopkins, undertook some experiments based upon these previous findings with the intention of making a thorough histological study of the eye lesions. The experiments were not done in the same building as were the earlier ones, but other conditions were precisely similar. The weight of the animals fell as usual, but no eye disease at all was obtained with purified artificial diets identical with those previously used, and after many weeks the experiment was given up.

Save in the case of the experiments done by Hopkins I have no detailed information as to the similarity of the symptoms observed by the different workers, nor as to the rapidity with which the xerophthalmia develops. In Hopkins' 1913 experiments, as already stated, it appeared as early as the fourteenth day. It is perhaps legitimate to assume that in the other cases cessation of growth, or initial absence of growth, marked the point at which the condition of the eyes became pathological, and that on resumption of growth the eye disease abated and ultimately cleared up. This, at any rate, seems to be the correct conclusion to draw from the evidence available. My own experience in the matter which has led me to the view already expressed is the following:

Since January, 1918, to the present date I have had approximately 500 young rats on varying experimental diets. To all these diets has been added water-soluble *B* in the form of a fat-free alcoholic extract of a yeast preparation. About 50 per cent. of the animals have received a supply of fat-soluble *A*, the remaining 50 per cent. having been on diets where fat-soluble *A* has been either deficient or absent. As regards this latter statement a diet has been considered "deficient" in fat-soluble *A* when young rats, receiving a proper supply of the water-soluble accessory substance, have failed to grow after, and often long before, the eighth week of the experiment. They have been supposed completely lacking in the factor when lard has been the source of fat supply. The basal diet of all foods fed has consisted of extracted caseinogen, unextracted starch, sugar and salts. The stock animals from which the experimental animals have been selected have been fed on bread and milk and oats, with the addition for the last four months of various vegetables.

During the whole of the period five cases only of xerophthalmia have occurred. Two of these were rats which were being fed on margarine with which was incorporated an unknown quantity of egg yolk as the source of fat-soluble *A*; one animal was on basal diet plus lard plus 2 cc. of milk per day; another was being given fatty acids in place of fat, and the remaining rat was a stock animal which had never been on any diet save the normal stock food.

The two first-mentioned animals were given butter as an addendum to their diet when the disease was well established, and their eyes were treated daily with boracic lotion. Both animals became cured within a week. Their growth curve was poor before the addition and good after. The animal having 2 cc. milk daily did not clear up at all and ultimately died; the one on fatty acids as its source of fat was fed with an addendum of butter and began to get better after a week. (This animal is still under observation and is not completely cured yet.) The rat on stock diet was a considerable time recovering—some five or six weeks—but did so ultimately. Thus of the five cases,

Two occurred on a diet where fat-soluble *A* was supplied, if to a limited extent.

One occurred on a diet where fat-soluble *A* was presumably present in sufficient quantity.

One occurred on a diet where fat-soluble *A* was probably absent.

One occurred on an ample stock diet.

Again,

Two animals were growing moderately well.

One animal lost in weight.

One animal neither declined nor increased in weight.

Each experiment lasted some three or four months and in each experiment there were at least eight other animals on the respective diets, showing no symptoms at all. Xerophthalmia on this evidence would appear to have no direct relation to diet, since these five animals were on different types of food; and to have little relation to growth.

It may be objected that the basal diet was not entirely free from fat-soluble *A* or that the fats used contained a sufficiency of the essential factor to prevent the onset of the disease. In view of the fact that practically all the animals on fat-soluble *A* deficient diets, though having an abundant supply of water-soluble *B*, failed ultimately to grow normally, and in many cases to grow at all, the former factor, if there at all, must, according to current views, have been present only in negligible quantities.

In addition to the above experience there remains that of Totani, who was using more highly purified foods and always lard as his source of fat. I consider that the discrepancy between Hopkins' and Totani's results, in which the animals were upon a diet absolutely identical in each case, is due to there having been the infection present in the former case and not in the latter, and that the freedom of my own animals from this disease is owing

(1) to the fact that no trouble has been spared to keep the animals under the very cleanest and healthiest conditions, and

(2) that care has always been taken to treat very promptly with boracic lotion any cases of slightly sore-looking eyes.

As regards the clearing up of the symptoms, which the quoted workers have observed on the addition of fat-soluble *A* it is surely probable that, as in so many cases of obstinate infection, general improvement of health brings about a cure of the local condition. I feel convinced that with avoidance of initial infection, experimental animals can be kept almost entirely free from this so-called deficiency disease, whether fat-soluble *A* be present or absent, and that it is dangerous to draw conclusions as to the fat-soluble *A* content of any diet from the appearance of xerophthalmia.

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XIII. THE PREPARATION OF SILICA JELLY FOR USE AS A BACTERIOLOGICAL MEDIUM.

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(Received March 6th, 1919.)

DURING the course of experiments involving the cultivation of seedlings in nitrogen-free media, a supply of silica jelly was required, free from combined nitrogen and of such consistency that it would support the growth of seedlings in tubes.

Preparation of the jelly according to published methods [Smith, 1905] failed to give satisfactory or uniform results, especially with regard to the consistency of the gel, although great care was taken at every stage of the preparation. Since other workers seem to have experienced similar difficulties, it was thought that an attempt to standardise the method of preparation of silica jelly might prove useful and possibly save much labour. The present note is the outcome of such an attempt and is published in the hope that it may be of service to other workers.

Preliminary work showed that the critical stages occurred during dialysis and that it was necessary to work with membranes of permeability varying within certain limits in order to obtain a uniform gel after autoclaving.

Collodion membranes, prepared and graded, as described by Brown [1915], were tried for dialysing, but it was subsequently found easy to prepare membranes of the requisite permeability without alcoholic treatment.

A solution of collodion is prepared by dissolving 6 g. of clean white guncotton in 100 cc. of a mixture of equal parts of absolute alcohol and ether. A quantity of not less than 500 cc. is made up and is allowed to stand for at least 24 hours to dissolve. The solution is then decanted into a bottle of convenient size and shape for holding in the hand. Should minute insoluble particles be noticed on the side of the wetted flask, due to unequal nitration of the guncotton, the solution is decanted through a filter of glass wool. These insoluble particles are the cause of fragile and leaky membranes. Filtered collodion is always more satisfactory provided that precautions are taken to prevent undue loss of ether by evaporation. It is impracticable to prepare a solution of collodion of definite composition, but for the purpose of making membranes it should be of a consistency similar to that of a heavy oil.

Test tubes $7\frac{1}{2} \times 1\frac{1}{2}$ inches were found most serviceable for the work. They must be thoroughly clean and dry, and also smooth; tubes with projections on the glass give rise to leaky membranes.

A test tube is held in a slanting position in the hand and slowly rotated while the collodion is poured down the inside until it is completely coated and a depth of about one inch has collected at the bottom. The collodion is then poured *slowly* back into the bottle, the tube being rotated as before. As soon as all the mobile collodion has entered the bottle, the tube is quickly inverted and its mouth placed firmly on a piece of paper with a folded duster, or pad of some kind, underneath, so that the tube is completely closed and an ethereal atmosphere maintained inside while it is allowed to drain for exactly 6 seconds. The accumulation of collodion is quickly wiped off and the membrane rapidly dried by passing a glass tube, attached to a filter pump, up and down the test tube, which is rotated horizontally, meanwhile, to keep the coating of collodion evenly distributed. During this drying process the finger is lightly pressed against the collodion a little way inside the mouth of the tube every eight or ten seconds. Immediately the collodion ceases to adhere to the finger the test tube is quickly and carefully filled with water, and allowed to remain full for at least three minutes to prevent further loss of ether.

It is important that the drying process should not be carried too far; the object in view being to obtain a thick membrane of low permeability; it may take any time from 30 seconds to 4 minutes according to the temperature and humidity of the laboratory.

The membrane is now detached from the mouth of the tube by the aid of a needle and removed by allowing water to pass between it and the glass aided by gentle pressure of the fingers.

These membranes are slightly milky in appearance, *i.e.* of the ether rather than the alcohol type, and are sufficiently strong for easy manipulation. They must be kept under water and never allowed to become dry.

For the dialysis of silicic acid a membrane of this size, when filled with distilled water and suspended in air, should not allow the water to drop oftener than once in twelve seconds. Membranes have been successfully used, with little difference in the time taken for dialysis, which had a range of from 1 drop in 12 seconds to 1 drop in 80 seconds; but the thicker membranes are more reliable and may be used many times.

Pure re-fused sodium silicate was used for the preparation of nitrogen-free media. This is supplied in opaque lumps which are slowly soluble in boiling water, but are readily brought into solution when raised to a temperature of 140° for one hour in the autoclave. 100 g. of sodium silicate in a litre of distilled water is readily diluted to give a solution of specific gravity 1.09.

A standard solution of re-distilled hydrochloric acid of specific gravity 1.10 is now prepared. It requires approximately 530 cc. of distilled water to the litre of acid to reduce the concentrated acid to this density.

Equal volumes of the acid and silicate solutions are taken, the former is poured into a conical flask and the latter into a large separating funnel and dropped into the acid which is shaken meanwhile. This acid mixture is allowed to stand from three to four hours, according to the temperature of the laboratory, in order that the necessary chemical reaction may take place, the longer period being given when the temperature is below 15° . The mixture is then poured into a separating funnel and thence into the membrane, which is supported in a vessel of water to avoid undue strain. When nearly full the neck of the membrane is drawn together and secured with a rubber band and the membrane suspended in a gas cylinder—or other suitable vessel—of distilled water to dialyse. The distilled water is changed hourly during the day and dialysis continued until the silicic acid is neutral to litmus. This usually takes two days. After the first twelve hours the water in the dialyser is tested with litmus paper before each change, which gives an indication of the state of acidity of the solution inside the membrane and avoids unnecessary manipulations.

Running tap water as supplied to the town of Reading was repeatedly tried for dialysis, but always caused the silicic acid to gel in the membrane before dialysis was complete.

When neutral to litmus, the silicic acid is poured into a beaker, the nutrient salts added in concentrated solution, and the medium boiled for five minutes to expel air. If not sufficiently boiled the plugs will be blown out of the tubes in the autoclave and an uneven gel will result. The medium is "tubed" whilst hot and immediately autoclaved for 20 minutes at from 135° — 140° .

Silicic acid prepared in this way forms a gel on autoclaving; if slants are required the tubes must be placed in the requisite positions in the autoclave as the gel once formed is irreversible.

The success of the process depends upon:

(1) The use of a membrane of standard permeability. A rather thick membrane of low permeability gives the best results.

(2) A sufficiently long period being given for the sodium silicate and hydrochloric acid to react after mixing. This is a critical factor.

(3) The use of distilled water for dialysing. Constant changes of water especially in the earlier stages of dialysis in a vessel just large enough to take a membrane, are best.

(4) Tubing the medium as quickly as possible after removal from the membrane and immediate autoclaving. The autoclave should be heated up while the medium is being tubed.

If it is not convenient to tube and autoclave the medium immediately after dialysis, it may be kept for about 24 hours in the dialyser. If allowed to stand for a short time in a beaker after boiling, dialysed silicic acid will probably form needle-shaped crystals.

It should be noted that compounds of silicon form deposits on glass.

These deposits are difficult to remove. It is therefore advisable to restrict the use of glass apparatus within reasonable limits during the preparation of the jelly.

In conclusion I wish to thank Dr M. C. Rayner for suggesting this work, and also for giving me much valuable advice and criticism.

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XIV. ELECTRICAL CONDUCTIVITY AS A MEASURE OF THE CONTENT OF ELECTROLYTES OF VEGETABLE SAPS.

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IN the course of some work involving a comparison of various methods of extracting saps, it has been found necessary to give attention to conductivity measurements as a means by which the content of electrolytes of samples of sap might be compared. A considerable amount of recent work in plant-physiology has been based on determinations of this nature in which it has been generally assumed that the conductivity of saps can be employed as a direct, though possibly somewhat approximate, measure of the content of electrolytes. It is clearly of importance that this assumption should not be made without adequate investigation, but hitherto no such investigation appears to have been carried out. On the other hand there are grave reasons for denying the validity of the proposition, even as a first and very rough approximation except under certain strictly limited conditions. Attention was first called to this point by an examination of the results of the conductivity measurements made by Dixon and Atkins [1913] on fruit juices. These were found to be uniformly low and the observers point out that this is the case even where acid fruits are in question, the juices of which are known to contain large quantities of organic acids. They explain their results as a consequence of the relatively slight dissociation of organic acids, but this explanation cannot be accepted as sufficient, as will be shown in detail below. It therefore became necessary to examine the matter further.

Since all fruits contain considerable quantities of sugars, it was thought possible that the presence of these in the juices might lower the conductivity and thus account for the discrepancies observed. This supposition was confirmed by comparative determinations of the conductivities of certain electrolytes (potassium chloride and citric acid) in aqueous solution and in solutions containing glucose, from which it was evident that the reduction of conductivity is considerable and must be of importance in the case of sugary saps.

It was subsequently found that the influence of non-electrolytes on the conductivity of aqueous solutions of various electrolytes had been worked out in detail by Arrhenius [1892] for a number of typical substances—electrolytes and non-electrolytes—and the object of the present paper is to discuss the bearing of the results thus obtained on the determination of conductivity in plant juices, especially in relation to the estimation of the content of electrolyte as carried out by Dixon and Atkins [1913].

Arrhenius found that for concentrations of non-electrolytes up to 10 per cent. (by volume) the conductivity of a solution may be expressed by the equation

$$C = C_0 \left(1 - \frac{ax}{2}\right)^2$$

where

C = conductivity of the solution containing x per cent. (by volume) of non-electrolyte;

C_0 = conductivity of an aqueous solution of the same electrolyte at the same concentration, and a = a constant.

The constant a varies with the nature of the non-electrolyte and of the conducting solution. It increases with the concentration of electrolyte but the variation is very small for highly dissociated substances. Arrhenius distinguishes the following groups of electrolytes. In each group the value of a is approximately constant for a given non-electrolyte.

Group 1. Strong univalent acids and alkalies.

Group 2. Salts giving univalent anions and cations.

Group 3. Salts giving univalent cations and bivalent anions.

Group 4. Salts giving univalent anions and bivalent cations.

Multivalent acids and weak univalent acids usually give still higher values of a and the same is true of salts which only undergo slight dissociation, *e.g.* HgCl_2 . It is to be noticed that if $\frac{ax^2}{4}$ which is very small be neglected

$$ax = \frac{C_0 - C}{C_0}.$$

The decrease in conductivity is therefore proportional to the concentration of non-electrolyte.

It has been already remarked that the concentration of a highly dissociated electrolyte can be varied within wide limits without producing more than a slight variation in the value of a . It is therefore impossible to find a general explanation of the reduction of conductivity produced by the addition of non-electrolytes by assuming a change in the degree of dissociation and this conclusion is supported by other considerations. Arrhenius attributes the greater part of the reduction to the increase of viscosity produced by the replacement of water by non-electrolyte, and he shows that for any given non-electrolyte a may be expressed with fair accuracy as a linear function of its viscosity in 1 per cent. solution. There is, however, some divergence due to change in the degree of dissociation and in the case of slightly dissociated substances

this factor cannot be neglected. It is to be noticed that the constants of the equation connecting a with the viscosity of the solution vary with the nature of the non-electrolyte. It is, therefore, impossible to find a general formula expressing C_0 in terms of C —the measured conductivity—and the viscosity.

The following examples taken from Arrhenius' tables will give some idea of the values obtained for a .

Table I. *Values of $a \times 100$ for various non-electrolytes.*

Non-electrolyte	Group 1	Group 2
Methyl alcohol	1.62	1.75
Ethyl alcohol	1.88	2.34
Isopropyl alcohol	2.03	2.56
Ether	1.63	1.99
Acetone	1.56	1.62
Cane sugar	2.44	2.99

Table II. *Values of $a \times 100$ for electrolytes of various constitution.*

Electrolyte	Ethyl alcohol	Cane sugar
Group 1	...	1.88
„ 2	...	2.34
„ 3	...	2.51
„ 4	...	2.39
H ₂ SO ₄ N/4	...	2.55
H ₂ SO ₄ N/200	...	2.53
Oxalic acid N/8	...	2.61
„ „ N/100	...	2.10

These values represent the percentage decrease in conductivity in a 1 per cent. (by volume) solution of non-electrolyte. By dividing by the density of the non-electrolyte, a more convenient value, a , is obtained which represents the percentage decrease of conductivity in a solution containing 1 g. of non-electrolyte in 100 cc. Table III gives values of a .

Table III. *Values of $a \left(= \frac{100a}{d} \right)$ for electrolytes of various constitution.*

Electrolyte	Ethyl alcohol	Cane sugar
Group 1	...	2.38
„ 2	...	2.96
„ 3	...	3.18
„ 4	...	3.03
H ₂ SO ₄ N/4	...	3.23
H ₂ SO ₄ N/200	...	3.20
Oxalic acid N/8	...	3.30
„ „ N/100	...	2.66

d for ethyl alcohol = 0.79.

d for cane sugar = 1.59.

It is now possible to estimate the correction which must be made in solutions containing non-electrolytes in order to obtain the corresponding conductivities in aqueous solution from the observed conductivities. Arrhenius'

equation may be taken as accurate up to 10 per cent. (by volume) of non-electrolyte. At 20 per cent. the error in the ratio $\frac{C}{C_0}$ is 4 per cent. Therefore in the case of plant juices the reduction of conductivity may without serious error be taken as proportional to the content of non-electrolytes. For cane sugar solutions the value of α is approximately 2; and glucose produces a very similar effect; the conductivity of a solution containing 5 per cent. of sugar is therefore reduced by 10 per cent. and the factor for the correction of the measured conductivity is $\frac{100}{90} = 1.11$. Since, however, many saps contain sugars and other non-electrolytes in much greater concentration than 5 per cent. the correction in these cases may be very large. The magnitude of this effect appears to have been much underrated in various recent researches on the conductivities of vegetable saps even where it is not altogether ignored. The work of Dixon and Atkins is a case in point, and it is interesting to calculate the conductivities of aqueous solutions corresponding to the saps investigated by them in cases where an approximate estimate can be made of the nature and quantity of the non-electrolyte present. For this purpose the values of C may be taken from a table compiled by Atkins [1916, p. 157] from results obtained by him and Dixon. They are shown below in Table IV compared with the corresponding values of C_0 .

Table IV.

Fruit	Concentration of sugar assumed	$C \times 10^5$	$C_0 \times 10^5$
	%		
<i>Citrus aurantium</i>	9	208	254
<i>Citrus limonum</i>	2	345	359
<i>Lycopersicum esculentum</i>	4	457	497
<i>Pyrus malus</i>	12	161	212
<i>Vitis vinifera</i>	14.5	112	158

Vaccinium oxycoccus has been omitted, as no estimate of its sugar content could be obtained. The sugar content of the other fruit juices is taken in round numbers from Wehmer's *Pflanzenstoffe* and C_0 is calculated from the equation

$$C_0 = \frac{100C}{100 - \alpha}, \alpha \text{ being taken equal to 2.}$$

The sugar content of fruits is of course very variable, therefore no approximation to accuracy can be claimed for the above figures; they serve however to show the order of magnitude of the error which must be produced by the sugars present in the juices; this amounts in the case of grapes to 40 per cent. It must be remembered too that the sugars are not the only source of error; the effect of alcohols, esters, pectins, dextrans and the remaining non-electrolytes other than sugars which are present has been ignored in the above calculation. In some cases these must play an important part, and a further

examination of one or two of the foregoing results may throw light upon this point besides providing criteria by means of which one may judge certain conclusions which Dixon and Atkins have drawn from their observations.

These conclusions are as follows: (1) that the influence of organic salts and acids on the conductivity is small [1913, p. 439] and (2) that "even in acid fruits by far the larger porportion of the osmotic pressure is still due to non-electrolytes" [Atkins, 1916, p. 157].

These statements as they stand are obviously difficult of acceptance and in the case of (2) the underlying fallacy is evident. Dixon and Atkins have made without further enquiry an assumption which is expressed by Atkins as follows: "that by finding the freezing point of solutions of some standard substance, such as potassium chloride, which have conductivities equal to those of the plant juices under examination, and by subtracting these values from the freezing points of the saps as actually observed, it is possible to get a very approximate measurement of the proportion of the osmotic pressure due to non-electrolytes. The values thus obtained are probably somewhat too low owing to the increased viscosity of the sap occasioned by the sugars in solution" [Atkins, 1916, p. 149]. This is in effect to assume a rough proportionality between osmotic pressure and electrical conductivity for which no theoretical basis exists, osmotic pressure being determined by the joint concentration of molecules and ions while electrical conductivity is a function of the number and velocity of the ions alone. How far astray the assumption may lead can be shown by examining the values obtained by Dixon and Atkins for lemon juice. Lemon juice is stated by Wehmer to contain 5 to 10 per cent. of citric acid; therefore the lemon juice examined probably represented at least a normal solution (6.4 g. in 100 cc.), the freezing point of which has been found to be -0.72° whereas by Dixon and Atkins' method the depression of the freezing point due to all the electrolytes in lemon juice works out at 0.170° . Indeed since the value of Δ for lemon juice was found by Dixon and Atkins to be 1.089° , and that for the acid it contains exclusive of salts has been shown to be of the order 0.7 it is clear that the depression of freezing point due to non-electrolytes must be very small. The conclusion stated above must, therefore, be reversed in this case. In lemon juice only a very small part of the osmotic pressure can be due to non-electrolytes.

Only a small part of this error can be attributed to the lowering of the conductivity by viscosity; the error is the natural result of the assumption that equi-conducting solutions of such substances as citric acid and potassium chloride contain an equal number of molecules and ions. In the case of a mono- or di-basic organic acid the error would probably be greater still. Where salts are in question the matter may be somewhat improved, since the degree of dissociation is rather more comparable with that of potassium chloride, but large differences still remain, and added to these is the effect of widely

divergent ionic mobilities, for in this case there are no mobile hydrogen ions present to compensate for the slowly moving anions. There is therefore no theoretical justification for the assumption and as the basis of an empirical formula it is far too precarious to be of any value even in the most limited field.

The conclusion drawn by Dixon and Atkins that the influence of organic salts and acids on the conductivity is small is based on the low conductivities which they observed. It has been shown above that these low results are to some extent a consequence of the presence of non-electrolytes in the saps, but in highly acid saps another very important factor must also be allowed due weight. It is well known that the hydrogen ion concentration of an acid solution may be greatly lessened by the addition of a salt of the same acid. In the case of organic acids possessing heavy slowly moving anions the conductivity is very largely due to hydrogen ions and in consequence the conductivity of a mixture of salt and acid may be less than that of the acid alone, especially where acid salts are formed. Thus the conductivity of a decinormal solution of citric acid was found to be 183×10^{-5} at 25° ; that of a similar solution which also contained $N/50$ sodium citrate was found to be 172×10^{-5} , while $N/100$ sodium citrate reduced the conductivity to 142×10^{-5} .¹

In these solutions where the principal electrolyte is an organic acid and where the degree of dissociation has been reduced by the presence of salts the action of non-electrolytes upon the conductivity becomes less marked and the value of α may fall very considerably below 2. The value of C_0 as calculated above will then be too high. The *joint* influence of organic salts and acids on the conductivity may thus under these conditions become relatively small and the assumption made by Dixon and Atkins be correct; but even where salts exert their maximum effect in reducing conductivity, the measured conductivity will still be very considerably below that of a corresponding aqueous solution if any considerable quantity of non-electrolyte is present.

It must also be observed that the values of α given by Arrhenius were in most cases determined for highly dilute solutions. These values tend to rise as the concentration of the electrolyte is increased, especially where, as for organic acids, the degree of dissociation of the electrolyte is largely reduced by increased concentration. This is an effect apart from and opposite to that produced by salts. Thus it will be seen from Table III that the effect of ethyl alcohol on the conductivity of oxalic acid is increased 24 per cent. when the concentration of the acid rises from $N/100$ to $N/8$. In order to ascertain the value to which α rises in highly concentrated solution, observations have been

¹ Since writing the above attention has been directed to two papers which bear upon this point. Haas [1917] gives measurements of actual and total acidity for a number of plant juices. Hempel [1917] examines the buffer effects produced by salts in the saps of various plants and the relation of P_H to titration in lemon juice.

made of the reduction of conductivity produced in solutions of normal citric acid by the addition of cane sugar and ethyl alcohol respectively. The solutions were made from ordinary commercial citric acid, equal volumes of 2N solution being diluted to normal. The results obtained are shown below.

Table V. *The effect on conductivity of additions of cane sugar and ethyl alcohol to normal solutions of citric acid.*

	$C \times 10^5$	Reduction of conductivity $\times 10^5$	α
Aqueous solution	553	—	—
Solution containing 2 % cane sugar ...	529	24	2.12
Solution containing 2.4 % ethyl alcohol...	497	56	4.21

The effect of alcohol on normal citric acid is thus nearly double that of cane sugar. Higher alcohols and esters appear to act in a similar manner and these may be of importance in reducing conductivity of the more acid fruit juices, but little is known as to the amount in which such substances are present in fruits.

There can be no doubt that the low conductivities which have been observed in fruit juices are due to two principal causes:

- (1) The action of non-electrolytes.
- (2) The mutual action of salts and acids in repressing dissociation.

Which factor will exert most influence must depend upon the nature and quantity of the acid present, the proportion of acid to salt and the concentration of non-electrolyte. Before it is possible to interpret the results of conductivity measurements in any given case further investigation is required of the influence of both salts and non-electrolytes upon the conductivity of organic acids, and also of non-electrolytes upon mixtures of these acids and their salts.

For the assumption that the influence of organic acids and salts on the conductivity is small and that the effect of non-electrolytes is usually negligible, Dixon and Atkins claim support from the results of certain experiments of Heald [1902] in which he found that the conductivity of certain plant juices was approximately equal to that of a solution of ash in a volume of water equal to that of the juice from which it was derived.

It must however be remembered that the relation of ash to sap is a very complex matter, and even were there no definite experimental evidence against the conclusion reached by Dixon and Atkins it would be hard to derive support for it from Heald's observations. The ashing of a sap involves a large number of changes which may affect conductivity. These include:

- (1) The destruction of organic acids.
- (2) The conversion of organic salts into carbonates some of which may be insoluble.

(3) The conversion of the metallic constituents of colloid substances (chlorophyll, proteins, pectins) into carbonates or oxides.

(4) The conversion of organic compounds of phosphorus and sulphur into phosphates and sulphates.

(5) The removal of non-electrolytes which reduce the conductivity.

How far these effects balance one another, must be largely fortuitous. In Heald's experiments the conductivity of the ash was usually found to be less than that of the sap, the difference varying from 2 per cent. to over 20 per cent. In the case of cucumber fruit—the only fruit investigated—the conductivity of the ash was only 60 per cent. that of the sap.

Dixon and Atkins' experiments on the effect of liquid air in rendering protoplasm permeable.

The foregoing considerations throw some light on an interesting series of experiments carried out by Dixon and Atkins [1913] on the differences in plant juices obtained by various methods. In these experiments a comparison was made between the juice from plant organs when pressed without previous treatment and the juice of the same organs when treated by exposure to liquid air before pressing. Determinations of freezing point and conductivity were made and on these were based conclusions as to the relative proportion of electrolytes and non-electrolytes present in each pair of samples. Dixon and Atkins found that in every case the freezing point of the juice from the treated organs was lower than that from the untreated, a clear indication that exposure to liquid air had reduced the resistance of the protoplasm to the passage out of the cell contents. They also found in many cases a corresponding but relatively less increase in conductivity which they interpret as showing that protoplasm in its untreated state is more permeable to electrolytes than to non-electrolytes.

In considering experiments of this nature it is desirable to form so far as possible some mental picture of the actual processes which take place. When living tissue is submitted to pressure it may be assumed that an exceedingly dilute solution of electrolytes will pass out at first, after which, as pressure is increased and a number of the cells are killed or burst, the greater part of their contents will escape. In the case of frozen tissues the cells of which are dead from the first, the escape of cell sap from the material will begin as soon as sufficient pressure is exerted. Dixon and Atkins assume that the difference in the value of the ratio $\frac{C}{\Delta}$ is evidence that a considerable quantity of electrolyte passes through the living protoplasm before it is killed by pressure. The interpretation of this ratio is, however, a matter of some difficulty and a further examination of Dixon and Atkins' work is necessary in order to draw definite conclusions from their experiments.

For this purpose a table compiled by Atkins [1916, p. 118] is quoted below which shows the effect of liquid air on the nature of the sap expressed from various plant organs.

				Δ	$C \times 10^5$	$\frac{C \times 10^3}{\Delta}$
(1)	<i>Hedera helix</i> leaves untreated	0.767°	403	5.2
	Same sample frozen	1.255°	605	4.8
(2)	<i>Ilex aquifolium</i> roots untreated	0.531°	563	10.6
	Same sample frozen	0.682°	629	9.2
(3)	<i>Ilex aquifolium</i> leaves untreated	0.651°	433	6.6
	Same sample frozen	1.130°	619	5.4
(4)	<i>Pyrus malus</i> fruit untreated	1.507°	171	1.1
	Same fruit frozen	1.919°	161	0.8
(5)	<i>Solanum tuberosum</i> tuber untreated	0.523°	555	11.0
	Same tuber frozen	0.588°	583	9.9
(6)	<i>Vitis vinifera</i> fruit untreated	2.567°	132	0.5
	Same sample frozen	3.185°	112	0.3
(7)	<i>Chamoerops humilis</i> leaf untreated	0.365°	298	8.1
	Same leaf frozen	1.529°	752	4.9
(8)	<i>Beta vulgaris</i> root untreated	1.473°	570	3.9
	Same root frozen	1.761°	555	3.2

It must be pointed out in the first instance that an increase in the total concentration of the constituents of the sap will not be accompanied by a proportional increase of conductivity, since the depression of the conductivity by non-electrolytes will also be proportionately increased. It is therefore necessary to consider in the first place whether Atkins was justified in the main conclusion which he drew from his experiments, viz. that the sap from the untreated tissue contained a higher proportion of electrolytes than that from tissue which had been previously frozen.

In order to obtain evidence upon this point the assumption is made here that the ratio of electrolytes to non-electrolytes in the two saps of each pair of samples from Atkins' table is the same, and that the relative decrease of conductivity in the sap from the frozen tissue is entirely due to the depression produced by the larger quantity of non-electrolyte it contains. On this hypothesis the amount of non-electrolyte can be calculated if a value be assumed for α . From Arrhenius's equation we have

$$\frac{C_2}{C_0} = \frac{100 - \alpha x}{100}$$

and

$$\frac{C_1}{C_0 p} = \frac{100 - \alpha p x}{100}$$

which gives

$$\frac{C_2 p}{C_1} = \frac{100 - \alpha x}{100 - \alpha p x}$$

where C_2 , C_1 = the conductivities of the saps from the frozen and unfrozen organs.

C_0 = the conductivity of an aqueous solution corresponding to the more concentrated sap.

p = the ratio $\frac{\Delta_1}{\Delta_2}$, which by hypothesis is the ratio of electrolytes and non-electrolytes in the two saps.

x = the percentage concentration of non-electrolyte in the more concentrated sap.

Below are tabulated the values of x calculated from this equation from the values of Δ and $C \times 10^5$ observed by Dixon and Atkins and quoted above; the value assumed for α is 2.

Table VI.

	Δ_1	Δ_2	$C_1 \times 10^5$	$C_2 \times 10^5$	x %	x^1 %*
<i>Hedera helix</i>	0.767°	1.255°	403	605	9	12
<i>Ilex aquifolium</i> roots ...	0.531°	0.682°	563	629	20	6
leaves	0.651°	1.130°	433	619	17	10
<i>Pyrus malus</i>	1.507°	1.919°	171	161	31	18
<i>Solanum tuberosum</i> ...	0.523°	0.588°	555	583	19	6
<i>Vitis vinifera</i>	2.567°	3.185°	132	112	35	30
<i>Chamocrops humilis</i> ...	0.365°	1.529°	298	752	23	14
<i>Beta vulgaris</i>	1.473°	1.761°	570	555	29	17

* x^1 represents the percentage concentration of hexose the osmotic pressure of which corresponds to Δ_2 . This is added for comparison. The corresponding quantity of cane sugar would of course be nearly double.

It must be remembered that equivalent conductivity will increase with dilution. In the case of acid saps this is very important, for the dissociation of most organic acids increases very rapidly as concentration diminishes. Increased dissociation may thus be largely responsible for the high value of x in such a case as that of *Pyrus malus*, or where the two samples of sap exhibit large differences of concentration.

Bearing these facts in mind a comparison of the values of x and x^1 does not appear to justify Dixon and Atkins' general conclusion that the proportion of electrolyte to non-electrolyte is higher in the saps from the untreated than in those from the treated tissues. In the majority of cases the differences observed in the ratio $\frac{C}{\Delta}$ can with great probability be accounted for by the fact that the electrolytes in the less concentrated saps are more highly dissociated and are subject to the action of a smaller quantity of non-electrolytes. In one or two cases, however, and notably those of *Ilex* and *Solanum*, it appears to be necessary to assume that there is an appreciable difference in the ratio of electrolyte to non-electrolyte in the two saps. This may be due to the expression of electrolyte from the untreated tissue before this becomes permeable to the other constituents of the cell sap as Dixon and Atkins assume, but it is clear that the same effect would be produced by the filtering out of non-electrolyte from the untreated tissue. If substance of high molecular weight were thus removed a considerable increase of conductivity might be produced while the accompanying change of osmotic pressure would be small and a small difference in concentration would accordingly account for the results obtained¹.

¹ It will be noticed that in three of the examples quoted from Atkins' table the conductivity of the sap from the frozen organ is less than that from the same organ untreated. It is suggested by Dixon and Atkins [1913, p. 430] that this is probably due to actual differences in the sap from different samples of the same organ, but that part of the effect may be due to viscosity. Differences of this magnitude would invalidate any conclusions that might be drawn from the experiments. It is however clear from the foregoing that it is entirely unnecessary to assume such differences.

One other point merits some further consideration. There is no doubt that the colloidal constituents of the sap tend to be filtered out in pressing and it is probable that the sap from treated tissues contains more of these substances than that from the untreated. It is therefore natural to suppose that this fact may provide an explanation of those cases in which the ratio of electrolytes to non-electrolytes varies in the two saps.

The case of *Solanum tuberosum* suggests the action of dextrin and it is natural to expect that pectin may exert a similar influence. There is, however, considerable doubt as to the part played by colloids in this respect, and Atkins has pointed out that the viscosity of solutions of this nature is not a measure of their influence upon conductivity. He instances the well-known case of gelatin gels and he describes an experiment in which a pectin solution was gelatinised and its viscosity therefore largely increased, while its conductivity remained unchanged. Atkins draws the conclusion that the effect of pectin upon conductivity is negligible and that this is true for the whole class of substances to which pectin belongs—this, however, is to go beyond the facts. Emulsoid colloids are two phase systems the two phases of which consist of solutions of the colloid in water and of water in the colloid. Conductivity appears to be conditioned by the composition of the more liquid phase only, but as this may contain a considerable quantity of the colloid the effect on conductivity may be far from negligible. All that Atkins' experiment appears to show is that the gelatinisation of pectin is a process which leaves the more liquid phase unchanged. It must be remembered, however, in regard to pectin that it has been observed to possess acid properties. Pectin and its salts may, therefore, have some conducting power in solution which will tend to counterbalance their effect on other electrolytes.

Until the action of colloidal substances has been further elucidated the complete interpretation of such observations as those of Dixon and Atkins cannot be attempted. It may however be stated with some assurance that their results indicate that in most cases the difference in the ratio of electrolyte to non-electrolyte in the saps from treated and untreated tissues must be very small, and that there is no evidence that any considerable quantity of electrolyte can be pressed out from the living cell. These conclusions are borne out by some experiments of André [1906, 1907] to which Atkins [1916, p. 119] has called attention. André determined the proportion of nitrogen and ash in samples of sap squeezed from various organs by successive pressures of 3, 12.5 and 25 kilos per square cm., and arrived at the conclusion that while the concentration of the substances present in the juice varied as the result of different pressures their relative proportions remained unchanged. The method was not susceptible of great accuracy. Small differences of concentration produced by filtration would scarcely be detected by this method especially if the substances filtered out contained neither mineral matter nor nitrogen. The experiments, however, lend some support to the view that any differences in the composition of the saps are mainly due to

the holding back of non-electrolytes by the untreated tissue in the course of filtration.

The foregoing analysis makes it clear that very great caution is needed in the interpretation of observations of the conductivity of plant saps. Decrease of conductivity may be occasioned not only by the addition of non-electrolyte but even in some cases by the addition of electrolyte, while changes of concentration often produce large changes in equivalent conductivity. It is hardly possible therefore to regard electrical conductivity as being in any sense a measure of content of electrolyte. Comparative measurements must nevertheless be of great value in the study of plant saps where methods are available by which the corresponding conductivity in aqueous solution can be ascertained.

It remains therefore to consider the possibility of introducing a correction by means of which conductivity measurements can be reduced to standard conditions.

Some indication of content of non-electrolyte can be obtained from freezing point determinations, but these are of little value for the present purpose, since change of conductivity is proportional to the actual content of non-electrolyte and not to its molecular concentration, so that *e.g.* a hydrolysis of cane sugar might take place which doubled the osmotic pressure, without producing an appreciable alteration of the conductivity. In saps which contain considerable quantities of sugar density determinations are of more value for this purpose, but the following appears to be the simplest method of determining the value of C_0 for a given sap.

By diluting with water to twice the volume the conductivity of the electrolyte in water is approximately halved and αx becomes $\frac{\alpha x}{2}$. If the conductivity of the diluted sap be then measured we have:

$$C_0(100 - \alpha x) = 100 C_1$$

$$\frac{C_0}{2} \left(100 - \frac{\alpha x}{2} \right) = 100 C_2$$

where C_1 , C_2 are the measured conductivities.

From these equations the value of C_0 and αx should be obtained with fair accuracy in those cases in which the degree of dissociation varies very slightly with the dilution. When saps contain any considerable quantity of organic acids it is impossible to assume that the conductivity is halved by diluting to twice the volume. In these cases the matter is far more difficult especially as the degree of dissociation of the acid and consequently the effect of non-electrolytes upon it will be largely determined by the amount of salts present in solution.

Further study of the action of both salts and non-electrolytes upon the conductivity of organic acids is necessary before helpful suggestions can be made in this connection.

SUMMARY.

1. Attention is drawn to the effect of non-electrolytes on the conductivity of electrolytes and a paper of Arrhenius is summarised in which the effect is investigated and its magnitude given for various cases.

2. An examination is made of the causes of the low values obtained in conductivity measurements in fruit juices containing considerable quantities of organic acids. It is pointed out that these low results must be ascribed to the action of non-electrolytes and of salts, but that further investigation of the mutual action of these two factors is necessary.

3. An attempt is made to estimate the influence of non-electrolytes and of the changes in the degree of dissociation produced by dilution in certain results obtained by Dixon and Atkins when comparing the saps obtained from frozen and unfrozen plant tissues. It is suggested that there is very little evidence for the marked differences which they assume to exist in the proportional composition of the two kinds of saps. It is further suggested that these experiments afford no evidence that the protoplasm of the cells of tissue under pressure is permeable to electrolyte to any considerable extent.

4. A formula is suggested by means of which in certain cases conductivity measurements may be reduced to standard conditions.

I desire to express my indebtedness to Professor V. H. Blackman for much help and criticism.

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XV. CONTRIBUTIONS TO THE STUDY OF THE VEGETABLE PROTEASES:

I. INTRODUCTORY¹.

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ALTHOUGH the work that has hitherto been done on the proteoclastic enzymes and their actions is considerable, the great bulk of it has been carried out in connection with those enzymes which occur in the animal body. Comparatively little has been done on the vegetable proteases². This is possibly due to the fact that whereas the three types of animal proteases can be readily differentiated according to their origin, very different is the case with the

¹ This investigation was carried out at the South-Eastern Agricultural College, Wye, between 1912 and 1914. Publication was delayed owing to the author's absence on military service.

² Some confusion exists in the terminology of the proteoclastic enzymes. Euler [1912] calls them *carbamases* or *proteinases* and considers that animal pepsin is a *pepsinase* and trypsin a *tryptase*. Vines gives the name *peptase* to those enzymes which convert the higher more complex proteins into albumoses and peptones, and *creptase* to those that split up albumoses and peptones into simple amino-acids.

The custom is rapidly becoming general of giving an enzyme a name derived from that of the substrate on which it acts by changing the final letter or syllable into *ase*. Thus lactase is the enzyme which hydrolyses lactose, urease that which decomposes urea into CO_2 and NH_3 . According to this nomenclature the names used by Euler and by Vines are obviously incorrect for a tryptase would mean an enzyme which acts upon trypsin, an creptase one that hydrolyses erepsin, and so on. It would be advisable in order to bring the terminology of the proteoclastic enzymes into line with that of other enzymes to apply the term *protease* to all those enzymes whether animal or vegetable in origin which will attack any of the proteins or their intermediate decomposition products. Those which attack the higher proteins only (*i.e.* those similar to pepsin) would be called *proteinases*, whereas those which split up albumoses, peptones, etc. but not the higher proteins (*i.e.* those similar to erepsin) would be *peptases*.

Animal trypsin is anomalous in some respects and has both proteinoclastic and peptoclastic properties. It cannot therefore be classed either as a proteinase or a peptase but only (generally) as a protease. It is not impossible though that trypsin may ultimately be found to be separable into a proteinase and a peptase [Vernon, 1904, 1; Vines, 1909]. Similar considerations apply to the vegetable ecto-proteases, *e.g.* of *Nepenthes*, which are considered to be mixtures of proteinases and peptases.

This terminology (which will be adhered to in the following paper) of course in no way conflicts with, or renders unnecessary, the classical individual names pepsin, trypsin and erepsin for the three particular enzymes so designated. Thus pepsin and erepsin are two particular enzymes belonging respectively to the classes proteinases and peptases.

vegetable proteases because a classification of these is not possible according to either the localisation of the enzymes or the nature of the media in which they act.

Three types of animal proteases are known:

1. *Pepsin*, which attacks so far as is known all true proteins. The products formed consist of albumoses and peptones, *i.e.* the hydrolysis is incomplete, lower polypeptides and amino-acids not as a rule appearing. Pepsin occurs in the gastric juice of all the vertebrates examined with the exception of certain fishes and it exerts its optimum activity in faintly acid solution, being quite inactive in an alkaline medium.

2. *Trypsin*, which is active in alkaline or neutral solution only, resolves proteins (as well as albumoses, peptones, etc.) into simple polypeptides and amino-acids. It is found in the pancreatic juice of all vertebrates, as well as in insects, protozoa, sponges, worms and molluscs.

3. *Erepsin* resembles trypsin in actively decomposing albumoses, peptones, protamines and polypeptides into simple amino-acids, but differs widely from it in its inability to decompose higher proteins such as fibrin and albumin. It occurs in the intestinal juice and intestinal mucous membrane and appears to be the most widely distributed of all the proteoclastic enzymes. According to Vernon [1904, 1, 2] the peptoclastic activity of trypsin is due to an ereptic enzyme (pancreato-erepsin) associated with it, so that it is conceivable that trypsin may be really separable into a pepsin and an erepsin (or rather a proteinoclastic and a peptoclastic part).

The vegetable proteases appear to be of two kinds which differ

(a) in mode of occurrence; and

(b) in the relations which exist between their digestive activity and the reaction, *i.e.* acidity or alkalinity, of the media in which they act.

One of these, and apparently the smaller class, consists of the extracellular or ecto-proteases which are found in the secretions of certain plants, *e.g.* in the antiseptic almost protein-free sap of the leaf pitchers of *Nepenthes*, in the leaf glands of *Dionaea* and *Drosera* and the leaf edge of *Pinguicula*, and also in bacteria especially those forms capable of liquefying gelatin. According to Vines [1897] the ecto-protease of *Nepenthes* is inactive in neutral and alkaline solutions, but is very active at the natural acidity of the pitcher liquid and also in the presence of free acid (up to 0.3 % HCl). This ecto-protease decomposes proteins not merely into albumoses and peptones but into amino-acids as well, and is considered by Vines to be a mixture of a proteinase and a peptase. On the other hand the ecto-protease of *Drosera* is active in alkaline and neutral as well as in acid media; it would appear therefore that the plant ecto-proteases are less rigid than the corresponding animal ones in respect to their activity in acid or alkaline media.

The second and larger class of vegetable proteases comprises the intracellular or endo-proteases most of our knowledge of which is due to Vines [1904, 1905, 1906, 1908, 1909, 1910]. They occur more especially in germi-

nating and ungerminated seeds and more abundantly in oil-bearing than in starch-containing seeds, and particularly in *Cannabis sativa*, *Sinapis*, *Ricinus* and *Linum*. They also occur in certain juicy fruits (*Ficus carica*) and leaves (*Agave*) and in many fungi such as yeasts. They belong to two groups (a) proteinases¹ and (b) peptases¹.

(a) The *proteinases* break down the true proteins such as fibrin and albumin, the decomposition however proceeding only as far as albumoses and peptones, behaving in this respect like animal pepsin. They are readily soluble in NaCl-solution but only slightly so in water or 50 % alcohol. Their extraction from seeds by means of water is therefore slow, as salts, etc., are extracted first, the enzymes dissolving in the solution so produced. Their digestive activity is greatest at the natural acidity of the plant extract. With pure aqueous extracts the activity is strong in neutral and even in slightly alkaline solution, but a slight addition of mineral acid (0.05 % HCl) or of rather more organic acid (0.30 % citric) arrests the action. Increase of alkalinity does the same. In this respect they differ from animal pepsin and the ecto-protease of *Nepenthes*, both of which show their optimum activity in the presence of free acids. In this connection it is worthy of note that Fernbach and Hubert [1900] regard the primary (acid) and secondary (basic) phosphates present in malt extracts as determining the course of protein-digestion, the former promoting, the latter retarding it.

The endo-proteinases very rarely occur alone in the organs of plants where proteins are decomposed but are usually (according to Vines always) accompanied by peptases which correspond with the erepsin of the animal body, differing from it only in their more extensive reaction range in the direction of acidity. As Vines points out, the fact that we never find proteinases apart from peptases must be correlated with the significance of the proteoclastic enzymes in the plant economy: proteinases alone would never be able to carry protein-hydrolysis far enough, as they hydrolyse only as far as albumoses and peptones.

(b) The *peptases* are readily soluble in water and in aqueous solutions of neutral salts. Their digestive activity is exclusively peptoclastic and is especially associated with an acid medium. The reaction range is not known as "pure" solutions of peptase have not been experimented with and the presence of other substances materially affects the action of acids and alkalis upon the enzyme.

Practically all the work done so far on the plant proteases has been confined to those that occur in seeds and in certain juicy fruits and insectivorous plants. Very little work has been done on the enzyme content of other parts of plants. The present investigation was undertaken with the object of determining whether proteoclastic enzymes exist in the ordinary foliage leaves and other parts of certain common farm crops and if so how far they

¹ Called by Vines peptases and ereptases respectively. Cf. note on p. 124.

resemble in their action the seed proteases. The crops experimented with, some fourteen in number, were grown on the college farm of the South-Eastern Agricultural College, Wye, in the spring and summer of 1912, some in the ordinary rotations and some specially for the purpose of this investigation. They belonged to the following species:

Cereals:

Barley	<i>Hordeum sativum</i> (Pers.)
Oats	<i>Avena sativa</i> (L.)
Maize	<i>Zea mais</i>
Rye	<i>Secale cereale</i>

Leguminous plants:

Red Clover	<i>Trifolium pratense</i> (L.)
White Clover	<i>Trifolium repens</i> (L.)
Lucerne	<i>Medicago sativa</i> (L.)
Sainfoin	<i>Onobrychis sativa</i> (L.)
Vetch	<i>Vicia sativa</i>
Field beans	<i>Vicia faba</i> (L.); <i>Vicia vulgaris</i> (Moench)
Peas (field var.)	<i>Pisum arvense</i> (L.)
„ (garden var.)	<i>Pisum sativum</i> (L.)

Other plants:

Buckwheat	<i>Polygonum fagopyrum</i> (L.)
White mustard	<i>Brassica alba</i> (Vis.); <i>Sinapis alba</i> (L.)

They were examined at two stages of growth: in full flower and at a comparatively young stage when the plants were about 8—10 ins. in height. The cereals, however, were examined in the grass stage only. They were gathered, dried and ground as finely as possible in a steel mill and only the part that passed through a sieve of 80 meshes per mm. was used for the work. Owing to the wet summer of 1912 it was found impossible to air-dry them sufficiently to admit of more than very coarse grinding; so recourse was had to oven-drying. The partially dried chopped material was placed in shallow trays in a large iron oven heated by means of Bunsen burners placed underneath to a temperature not exceeding 45°—50°. In these circumstances it is practically certain that the injurious effects of the increased temperature on the enzymes present would be a minimum. It is well known that diastase can withstand higher temperatures than this even in aqueous solution and in common with all enzymes can withstand much higher temperatures when *in situ* than when in solution; *e.g.* in plant material diastase can be heated to 80° without being destroyed although its hydrolysing power is somewhat impaired. Miss White [1909] observed that heating dry oats to a temperature of 100° for 4½ hours was without effect on the enzymes present although they were destroyed by heating to 130° for one hour. The present author has observed that heating dry linseed cake in a steam oven to a temperature of 99° for 4 hours caused only a small reduction (less than 10 %) in the activity of the cyanogenetic

enzymes present. It is true that proteoclastic enzymes are more sensitive to heat than is diastase particularly in solution, but both substrate and decomposition products exert considerable protecting action (Bayliss, Vernon). Moreover, the optimum temperature of animal protease is given as 40° . The presence of such large quantities of adsorbent material as are present in green plants combined with the comparative dryness would tend to make the enzymes still more resistant to heat; so that in all probability no more damage would be done than in the case of simple air-drying under glass. At the same time oven-drying at this temperature facilitates grinding, enabling a much finer powder to be obtained.

In the case of such crops as lucerne and sainfoin which lignify rapidly very early in the flowering period, the main woody stems were rejected and only the leaves, flowers and smaller and younger twigs retained.

Owing to the bulkiness and comparatively small proteoclastic activity of the plant material and the dark colour of the extracts considerable difficulty was experienced in devising a method for estimating any enzymic activity there might be.

The method followed by Vines was first tried. This consists in detecting the presence of tryptophan (a frequent product of tryptic hydrolysis) by means of the violet colour produced by bromine water in the presence of a little HCl. The method requires considerable care and experience in its use before it can be used successfully even with "pure" trypsin and substrate, *i.e.* in colourless or nearly colourless solutions. In the present case the *differences* in activity exhibited by the various plant materials used were insufficient to be detected—let alone estimated—by this method. It was, therefore, abandoned as a quantitative measure but was used as a qualitative test for proteoclastic action since in every instance the controls gave no tryptophan reaction.

The method finally adopted was Sørensen's [1908]. By this method the extent of protein-hydrolysis can be determined from the number of free carboxyl groups formed. By adding excess of formaldehyde solution the free amino-groups of the amino-acids produced were neutralised forming methylene compounds; the carboxyl groups can then be estimated by titrating with $N/5$ Ba(OH)₂, using thymolphthalein as indicator. In this way the extent of protein-hydrolysis can be expressed by the number of cc. of $N/5$ Ba(OH)₂ used in the titration. Also on the assumption that each carboxyl-group formed during the hydrolysis corresponds with one amino-group, the amount of hydrolysis can be stated as mg. of nitrogen, this being obtained by multiplying the number of cc. of $N/5$ Ba(OH)₂ required by 2.8.

In using this method two solutions are required:

A. 0.50 g. of Grubler's thymolphthalein dissolved in 1000 cc. of 93 % alcohol;

B. 50 cc. of commercial formaldehyde (40 %) + 25 cc. of absolute alcohol + 10 cc. of thymolphthalein solution *A.* $N/5$ Ba(OH)₂ is run in until a faint

green or blue colour is obtained. This solution must be prepared fresh for each series of experiments.

In practice 10 g. of the plant material + 250 cc. of distilled water which had been raised to 40° ¹ + a thin layer of toluene in a tightly corked flask served as a control.

10 g. of plant material + 5 g. of Witte's peptone + 250 cc. of water at 40° ¹ + a thin layer of toluene; and 10 g. of plant material + 3 g. of legumin + 250 cc. of water at 40° ¹ + a thin layer of toluene served as the experimental liquids. All three were contained in tightly corked flasks and were kept in an incubator at 37° for three days². At the end of that time they were filtered as rapidly as possible, and the residues were thoroughly washed with cold water until the volume of the filtrate and washings from each was 400 cc. Preliminary experiments had shown that all amino-acids were washed out when the filtrate and washings occupied this volume. A tenth, *i.e.* 40 cc., of each filtrate was then transferred to a flask, decolorised as far as possible by shaking with alumina cream³, filtered and the residue thoroughly washed. To the filtrate and washings from the control was added 15 cc. of solution *B* and $N/5$ $\text{Ba}(\text{OH})_2$ run in until a distinct blue or green colour⁴ was obtained. The other filtrates were then treated in the same way, $N/5$ $\text{Ba}(\text{OH})_2$ being added to them until their tint matched that of the control solution. To the actual readings of $\text{Ba}(\text{OH})_2$ used a correction had to be applied to allow for the amount of $N/5$ $\text{Ba}(\text{OH})_2$ absorbed by the undecomposed protein, *i.e.* either legumin or peptone, left in the solution.

The results obtained for the action of 10 g. of plant material on 5 g. Witte's peptone during 3 days' incubation at 37° are given in Table I, A.

A consideration of the results given in Table I, A, indicates that a protease capable of splitting up Witte's peptone into simple amino-acids is present in all the plants examined. The peptoclastic activity varies with the nature of the plant but is an individual rather than a generic character. Thus, contrary to expectation, the leguminous plants examined did not on the whole possess greater peptoclastic power than the non-leguminous ones. The activity of the foliage of young peas (field variety) was found to be some $2\frac{1}{2}$ times that of young oat plants; but the leguminous plants other than peas were in no way

¹ This temperature was chosen in order that the temperature of the plant-water mixtures might be in the neighbourhood of 37° at the beginning of each experiment.

² This period was shown by preliminary experiments to be a suitable time for digestion. It was sufficiently long to allow a measurable amount of hydrolysis to occur but not long enough for the reaction to have reached its limit.

³ The alumina cream was made by gradually adding excess of ammonia solution to saturated alum solution with constant shaking, allowing to stand for several hours, and then filtering off the precipitated alumina. It is very important that this should be washed until free from NH_3 and sulphate either of which would interfere with the titration. The alumina was then shaken up with water until a thin cream was obtained.

⁴ Blue if the original solution was colourless, green if it was yellowish or brownish. Most of the extracts dealt with were deep brown in colour and after shaking with alumina cream were slightly yellow or brownish. This did not interfere with the titration provided all three filtrates were of the same tint.

superior to the non-leguminous ones, while white mustard (a non-leguminous plant) especially in the flowering stage was distinctly more active than any of the other plants examined except peas.

Table I.

Name of plant	A. <i>Witte's Peptone</i> ¹ .		B. <i>Legumin</i> ² .	
	N lib. in mg. c.c. $N/5$ $Ba(OH)_2$ required for whole extract $\times 2.8$	Percentage of peptone hydrolysed	N lib. in mg. = c.c. $N/5$ $Ba(OH)_2$ required for whole extract $\times 2.8$	Percentage of legumin hydrolysed
Cereals (grass stage only):				
Barley	128.80	18.68	30.66	5.68
Oats	78.40	11.37	18.93	3.51
Maize	103.60	15.02	37.80	7.01
Rye	109.20	15.84	16.80	3.11
Leguminous plants:				
Red clover (young)	135.80	19.70	24.92	4.62
„ (old)	166.60	24.16	35.28	6.54
White clover (young)	134.40	19.49	22.68	4.20
„ (old)	165.20	23.96	30.24	5.61
Lucerne (young)	85.60	12.41	21.28	3.94
„ (old)	158.50	22.99	25.56	4.74
Sainfoin (young)	98.00	14.21	8.93	1.65
„ (old)	152.00	22.04	10.22	1.89
Vetch (young)	120.40	17.46	19.15	3.55
„ (old)	144.20	20.91	24.27	4.50
Beans (young)	81.20	11.78	10.22	1.89
„ (old)	102.20	14.82	15.34	2.84
Peas (f. var.) (young)	196.00	28.43	38.07	7.05
„ „ (old)	236.60	34.31	50.10	9.36
Peas (g. var.) (young)	140.00	20.30	39.20	7.27
„ „ (old)	177.80	25.79	47.88	8.88
Other plants:				
Buckwheat (young)	89.60	12.99	7.675	1.42
„ (old)	112.56	16.32	8.50	1.57
White mustard (young)	125.20	18.16	17.92	3.32
„ (old)	199.40	28.92	25.56	4.74

¹ Percentage of nitrogen in Witte's peptone = 13.79.

² Percentage of nitrogen in legumin = 17.97.

The activity varies with the stage of development of the plant and is in every case greater at the flowering period than in the earlier ones.

In some cases the tryptophan reaction was applied and gave positive results in some few instances with the plant-peptone extracts; with most of the plant extracts, however, as with the controls, no colour change occurred. It does not follow of course that no tryptophan was formed in these plant extracts either from the splitting up of the plant proteins themselves or from the added protein. In any case very little would be formed and this would be distributed throughout 400 cc. of liquid of which 10—20 cc. were taken

for the test. The absence of any tryptophan reaction is therefore quite explicable even though some slight formation of tryptophan had occurred.

In Table I, B, are given the results obtained for the action of 10 g. of plant material on 3 g. of legumin during 3 days' incubation at 37°.

The results given in Table I, B, are interesting in view of Vines' statement that out of a very large number of leaves examined by him only those of the lettuce and of *Phytolacca decandra* would digest blood fibrin or milk caseinogen. All other leaves gave either a doubtful or negative result. From this he concluded that the hydrolysis of albumoses and peptones by green plants is related to the fact that those proteins which occur naturally in the plant are such as are readily digested; whereas the more resistant ones similar to fibrin and caseinogen (which possibly do not occur in plant tissues, but only in the seeds where proteinases do undoubtedly occur) are not attacked.

The two results are, however, not altogether incompatible. Vines used fibrin in the form of small shreds in his experiments and relied on the appearance or, rather, the disappearance, of the fibrin after incubation as a test of hydrolysis. The legumin used by the present author was in the form of a very fine powder; the surface exposed to any proteoclastic action there might be would be much larger than in the former case and digestion therefore more rapid. The method employed for detecting the hydrolysis was also a more delicate one and even under these favourable conditions the hydrolysis was slight as compared with that of the peptone. It is doubtful whether such a small hydrolysis would produce any *visible* effect on small shreds of fibrin suspended in the extracts.

That the activity indicated is enzymic in nature and not merely the result of imperfections in the conditions of experiment seems to be shown by the fact that, as with peptone, the activity is invariably greater at the flowering stage than at the earlier one, and the greater activity of peas is again evident when legumin is employed as the substrate.

Having established the presence of proteinoclastic and peptoclastic enzymes in all the green plants examined it was next decided to examine one or two plants in detail in order to find out how the different parts of the plants varied as regards enzyme content. For this purpose an examination was made of beans, peas (field variety) and buckwheat. In this portion of the work the different parts of the plant were gathered separately and dried—first by air-drying and then *in vacuo* over sulphuric acid.

To test the effect of germination the seeds were germinated in moist sand kept in a warm place, were dried as rapidly as possible in an air-oven at 40–45°, coarsely ground and finally dried *in vacuo* over sulphuric acid. The enzymic activity was determined by Sørensen's method as described above. The results are given in Table II, A and B.

An examination of the tables shows that all parts of the plant are active and at all stages of growth. The activity of the leaves and seeds presents features of particular interest. Both the proteinoclastic and peptoclastic

activity of the leaves increases with increasing maturity right up to the time of harvesting and does not fall off, as was at first anticipated, after the flowering stage when translocation of foodstuffs from the leaves to other parts of the plant is taking place and during the dying off of the leaves. In this connection it is interesting that increasing activity with increasing maturity of the foliage right up to the time at which the leaves begin to die off has been shown to hold also for the cyanogenetic enzymes present in flax foliage. Thus the foliage of flax in full flower has been found to be rich in both cyanogenetic enzyme and cyanophoric glucoside; whereas flax leaves which have turned brown at the edges are still as rich in enzyme some time after the corresponding glucoside has completely disappeared.

Table II.

Part of plant	A. <i>Witte's Peptone.</i>		B. <i>Legumin.</i>	
	N lib. in mg. = c.c. $N/5$ Ba(OH) ₂ required for whole extract × 2.8	Percentage of peptone hydrolysed	N lib. in mg. = c.c. $N/5$ Ba(OH) ₂ required for whole extract × 2.8	Percentage of legumin hydrolysed
Beans:				
Leaves (plant 3-4 in. high)	81.20	11.77	10.22	1.73
.. (full flower) ...	102.20	14.82	15.34	2.84
.. (at harvest) ...	159.80	23.71	17.92	3.32
Flowers	153.40	22.25	19.18	3.56
Seeds (ungerminated) ...	154.65	22.43	14.06	2.61
.. (just germinated) ...	188.20	27.29	12.77	2.37
.. (germinated) ...	230.10	33.37	14.06	2.61
Pods	139.45	20.22	10.00	1.74
Stems	93.50	13.56	14.06	2.61
Peas (field var.):				
Leaves (plant 6-8 in. high)	196.00	28.42	38.08	7.06
.. (full flower) ...	236.60	34.32	50.96	9.45
.. (at harvest) ...	276.10	40.04	55.44	10.38
Flowers ¹	171.40	24.85	25.56	4.74
Seeds (ungerminated) ...	120.40	17.46	24.08	4.47
.. (germinated) ...	244.10	35.41	47.04	8.80
Pods	145.00	21.03	12.60	2.34
Stems	83.44	12.10	13.44	2.49
Buckwheat:				
Leaves (v. young) ...	89.60	12.99	7.67	1.42
.. (full flower) ...	112.55	16.32	8.95	1.66
.. (at harvest) ...	222.40	32.25	10.22	1.90
Flowers	122.60	17.79	35.78	6.64
Seeds (ungerminated) (6 dys.)	103.40	15.00	5.18	0.96
.. (just germinated) ...	114.80	16.65	12.77	2.37
.. (germinated) ...	177.90	25.21	—	—

¹ This figure is only approximate as the pigment present interfered with the titration and made the end-point indeterminate.

The effect of germination on the enzyme content is also of considerable interest. It would appear from the results obtained that ungerminated seeds,

like all other parts of the plant, are active towards Witte's peptone and slightly so towards legumin. When germinated so that the young shoot is just showing itself a distinct increase in peptoclastic activity occurs; when the germination has proceeded until the shoots are one to two inches in length both the proteinoclastic and peptoclastic activity become much greater. This is shown best in the case of buckwheat and falls in line with Vines' observations that the activity is not at once evident but is developed during the germination process¹.

The above investigation also throws a little light on another interesting point. Vines [1903] first pointed out the possibility of the proteinoclastic and peptoclastic enzymes of green fodder crops being of use to the animal during the process of digestion. He pointed out that in the case of herbivorous animals and especially the ruminants the vegetable food that has been eaten is probably placed under conditions that are altogether favourable to the action of the proteases which it contains, so that there is reason to believe that digestion in these animals is, in no small degree, a process of autolysis, the food providing at once the nutriment and the means of digesting it. It is by no means impossible too that the enzymes present may even assist in the digestion of any additional protein that may be fed along with the greenstuff. The results obtained above indicate that the peptoclastic activity of green fodder plants is sufficiently great to be of assistance to the animal in the digestion of the simpler, more easily digested, proteins, but that the proteinoclastic activity is much too slight to be a serious factor in the digestion of the higher proteins that may be present in, or fed along with, the green food.

Whether the conditions operative in the animal's stomach are such as admit of auto-digestion of proteins taking place is another matter and cannot be determined until we know the quantitative relationship that exists between the peptoclastic activity of plant proteases and the reaction of the medium in which they act. Attempts were made to determine this but with little or no success. Various aqueous extracts of the plant material were obtained and the action of acids and alkalies upon their peptoclastic activity was investigated. In the case of the alkalies more or less bulky precipitates were formed in all cases during the incubation and no consistent results could be obtained. This is not very surprising when it is remembered that plant extracts are very slightly acid in reaction. On changing the reaction of the medium the internal equilibrium is disturbed and the colloidal substances present are partially precipitated carrying some enzyme down with them.

The whole extract is an extraordinarily complex colloidal system and even

¹ The sudden increase in peptoclastic activity that occurs in seeds during germination finds an interesting parallel in the case of the lipase of linseed. While a lipase is frequently found in many oil-bearing seeds—being especially abundant in species of *Ricinus*—its presence had not until recently been detected in linseed although many attempts have been made to find it. The present author, in conjunction with Dr J. V. Eyre, has succeeded in showing the presence of a lipase in *germinated* linseed and has measured its activity. It would appear to be developed during the germination process.

if no disturbing factors were introduced and it were possible to determine accurately the effects of acids and alkalies on the enzymic activity the results obtained would not necessarily correspond to those obtained when using "pure" enzyme preparations. All that can be deduced from these latter experiments is that the peptoclastic activity of plant extracts is not destroyed by the addition of small quantities (*e.g.* up to 0.05 %) of either acids or alkalies. But the direct action of acids or alkalies cannot be determined until the enzymes can be extracted in a less impure condition. This is being attempted, and it is hoped that a systematic study of the vegetable peptases may form the subject of a further communication.

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XVI. ON THE ESTIMATION OF SUGAR IN BLOOD.

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IN spite of the large numbers of determinations of blood sugar that have been carried out by various means in the past few years there is still need for a reliable and simple method.

Much work has recently been done with the picric acid procedure in which the depth of colour produced is estimated by means of the colorimeter. It is, however, a drawback to this method that a colorimeter is not always available especially for clinical work. Further, it is doubtful whether this method is at all applicable in the case of whole blood on account of the creatinine (and probably other interfering substances present) which, under the conditions of the estimation, give a colour similar to that produced by glucose in the presence of picric acid. De Wesselow points out [1919] in the following paper that these interfering substances are always active and in the case of human blood may actually influence the picric acid method to such an extent that the result obtained may be as much as 50 % too high. In plasma also the method appears to yield results on the high side, but here the figures obtained are probably accurate enough for practical purposes.

Bang in 1913 introduced a micro-method for blood sugar determination, but the difficulty of completely separating the protein and the ease with which oxidation of the cuprous oxide took place before and during titration interfered very materially with its practical utility.

A new application in which the tendency to oxidation of cuprous oxide during titration was completely overcome was introduced by the writer a few years ago. This method was first used in 1914 and was communicated to the Biochemical Society in autumn 1915. A few months after this communication was given, a method based on the same principle was published independently by Scales [1915] while my paper was actually in the press [MacLean, 1916]. This method gives excellent results and the fact that Bang [1918] adapted this principle to his own method speaks for itself. The application of the method for the estimation of blood sugar was described by me in the above noted publication [MacLean, 1916]. An extended use of the procedure, however, has resulted in various improvements and considerable simplification, and it is felt that the modified method as described in

the present paper not only gives very reliable results, but is so simple in its application that it can be carried out by students and can easily be utilised for clinical purposes.

PRINCIPLE OF THE METHOD.

In estimating the sugar in blood it is first necessary to separate the protein very completely. For this purpose various procedures were tried, but the use of dialysed iron in conjunction with heat coagulation gave the best results. In the new method the blood is heated in an acid saline solution and the greater part of the protein separated by coagulation. Any remaining traces of protein are removed by the addition of a small amount of dialysed iron. After cooling, the mixture is filtered and a perfectly clear protein-free filtrate obtained. The sugar in an aliquot part of this filtrate is estimated by boiling the liquid with an alkaline copper solution containing potassium iodate and iodide. After boiling, the solution containing the reduced cuprous oxide in suspension is cooled and treated with a slight excess of hydrochloric acid. This interacts with the potassium iodate and iodide liberating iodine equivalent to the amount of potassium iodate in the solution. At the same time the cuprous oxide is dissolved and cuprous chloride formed. A solution of the latter is very unstable and liable to oxidation, but this is prevented by the fact that free iodine is present with which it immediately reacts. Since the cuprous oxide present in the mixture at an earlier stage is not in solution but in suspension, no oxidation takes place during the preliminary cooling. During the boiling, any air present in the solution is driven off before the sugar has time to reduce the copper to any appreciable extent; also the flask is full of steam during this time so that no oxidation from air oxygen need be feared.

The amount of sugar present in the solution is calculated by ascertaining the amount of iodine used up as fully described later.

The only point in the method that requires special care is the regulation of the flame used to boil the sugar and alkaline copper solution. The tables given in this paper are worked out for a flame which brings the amounts of solution specified later to the boil in as nearly as possible one minute forty seconds. In this time the *boiling should be fully established*; often some bubbles come off 10 seconds or so before the boiling becomes brisk, but it is usually quite easy to ascertain the point of complete boiling to within a few seconds. For clinical purposes a rough preliminary standardisation of the flame is all that is required, but for very accurate work the use of a small manometer is necessary. When care is taken to follow the directions given the results obtained vary only within the limits of the ordinary experimental error of manipulation which of course is a personal factor. With pure solutions of glucose successive identical readings are obtained with monotonous regularity if the conditions are carefully observed. With blood also duplicate experiments yield practically identical figures (see below).

One detail in the method requires some comment. When hydrochloric acid is added to the boiled mixture of alkaline copper solution and cuprous oxide the acid reacts with the carbonates present and effervescence takes place. Since free iodine is present at this stage it might be expected that this effervescence would result in driving off some of the iodine. That this may be the case to some infinitesimal extent is probable, but I have satisfied myself that when ordinary care is taken not to agitate the mixture too vigorously until the effervescence passes over no appreciable amount of iodine capable of estimation is lost. This probably depends on the extreme dilution of the iodine solution.

For blood sugar determinations the method is applicable to 1 cc. or smaller amounts and descriptions of the procedure for estimating sugar in 1 cc. and in 0.2 cc. of blood respectively are given here. The use of such a small amount as 0.2 cc. blood has various advantages, since this amount can be easily obtained from the finger. For 1 cc. it is necessary to insert a needle into a vein of the arm, and if observations are required at frequent intervals this procedure becomes difficult for obvious reasons. On the other hand, quantities of 0.2 cc. blood can be obtained from the ear or finger as often as may be required.

In all blood sugar estimations it is important to remember that glycolysis is often very active especially for a short time after the blood is drawn; for this reason the specimen should be immediately measured and added to the coagulating mixture described and, if convenient, heated at once. By this means glycolysis is completely prevented. After standing for two hours at room temperature the amount of sugar present in the specimen may be less than half its original sugar content. Since the method of obtaining 0.2 cc. blood described later allows of almost immediate mixing of the blood and coagulating fluid, the use of this small amount has an additional advantage.

Detailed instructions for the preparation of the various solutions required are given below. An objection urged against the method was the difficulty of preparing an accurate $N/10$ thiosulphate solution, but this difficulty no longer exists since standardisation against potassium bichromate is now used.

SOLUTIONS REQUIRED FOR 1 CC. METHOD AND THEIR PREPARATION¹.

The solutions required have the following composition and are prepared according to the directions given.

A. *Solutions for removing protein from blood.*

No. 1. *An acidified sodium sulphate solution.*

Sodium sulphate (pure)	150 g.
Glacial acetic acid	3 cc.
Distilled water to	1000 cc.

¹ All these solutions may be obtained ready for use from the British Drug Houses, Graham Street, London.

Solution should be filtered through a starch-free filter paper (Whatman No. 1).

No. 2. Dialysed iron solution.

This solution should be about B.P. strength and well dialysed. The majority of the samples on the market are practically not dialysed at all and contain very large amounts of chlorides. A suitable preparation is stocked by the British drug houses under the term "Dialysed iron B.D.H."

B. Solutions for estimating sugar in filtrate.

No. 3. Alkaline copper iodide solution.

Potassium bicarbonate	20	g.
Potassium carbonate (anhydrous)	15	,,
Copper sulphate crystals	0.7	,,
Potassium iodate	0.11	,,
Potassium iodide	1	,,
Distilled water to	100	cc.

In making up the solution 20 g. of potassium bicarbonate are dissolved by gentle heating in 60 to 70 cc. of distilled water. The heating must be very carefully done and the temperature of the mixture should not exceed 37°. The potassium carbonate is then added. The copper sulphate is dissolved in a separate beaker in a few cc. of water and added to the mixture of carbonates without waiting for the potassium carbonate to dissolve completely. After the resulting effervescence has passed over, the complete solution of any remaining carbonate is accomplished by heating. The iodate and iodide are next added, the solution thoroughly shaken and made up to 100 cc. After filtering through a starch-free paper (No. 1 Whatman paper) it is ready for use. The solution keeps indefinitely. The copper sulphate and potassium iodate must be weighed with great care. The other ingredients need not be so carefully measured.

This solution must be standardised as regards its iodine content by taking exactly 3 cc. of the mixture and adding this to 10 cc. of the 15 % sodium sulphate acetic acid solution used for deproteinising the blood. To this mixture 10 cc. of 20 % hydrochloric acid are added. The flask is very gently agitated until effervescence passes over and then more strongly agitated for 1 minute. A solution of N/100 sodium thiosulphate is run in until the yellow colour of the iodine has almost completely disappeared. Two drops of starch solution are then added and the titration proceeded with until the blue colour disappears. The number of cc. of thiosulphate used up gives the equivalent of iodine liberated from the mixture and should be recorded on the bottle. For 3 cc. copper iodide solution about 8.8 cc. of N/100 thiosulphate are required. The exact figure obtained depends to some extent on the purity of the samples of iodate and iodide used, but variations due to this should be very slight. The samples used in my work give exactly 8.82 cc. thiosulphate = 3 cc. copper solution.

No. 4. (*N/10 thiosulphate solution.*)

Though sodium thiosulphate crystals can be obtained in a state of great purity it is generally stated that the salt cannot be weighed directly for the purpose of making a standard solution owing to the possibility of water or air being held within the crystals. This difficulty may be avoided by treating the finely ground crystals with pure alcohol and then with ether and finally drying at room temperature. This manipulation, however, is tedious and there is always some slight risk that the crystals may not have been dried in air for a sufficiently long time to give an ultimate product containing the correct amount of water of crystallisation. The preparation of an accurate iodine solution for standardisation by weighing the iodine is also a tedious procedure. To avoid all these difficulties, the sodium thiosulphate solution used for this method is prepared by standardising against an *N/10* solution of potassium bichromate. In preparing the *N/10* thiosulphate a solution of *N/10* potassium bichromate is first prepared by dissolving 4.913 g. of the pure dried salt in distilled water and making up the volume to 1000 cc.

The distilled water used for the thiosulphate solution should be boiled immediately before use to get rid of traces of CO_2 . About 26 g. of pure sodium thiosulphate crystals are dissolved in about 1000 cc. of the cooled, boiled out water. To estimate the strength of this solution 20 cc. of *N/10* bichromate are mixed in a flask with 10 cc. of 10 % potassium iodide and 5 cc. of strong hydrochloric acid. On shaking slightly the reaction is complete and exactly 0.254 g. iodine is liberated. The thiosulphate solution is run in from a burette until the brownish yellow colour has almost disappeared. One or two drops of a starch solution are then added and the titration continued until the liquid passes from a dirty dark yellow to a bright green colour. The end point is very sharp and cannot be mistaken. The number of cc. of thiosulphate used up indicates the strength of the thiosulphate solution. Since, however, all samples of potassium iodide contain traces of iodates, the iodide solution alone without bichromate will yield a very small amount of iodine in the presence of acid. This must be allowed for by making a blank experiment with 10 cc. potassium iodide and 5 cc. hydrochloric acid and titrating with thiosulphate as before. Not more than 0.1 to 0.2 cc. of thiosulphate should be used up. This must be subtracted from the result obtained with the bichromate. If say 18.7 cc. thiosulphate were required and the potassium iodide gave a blank of 0.2 cc., then the amount of thiosulphate solution required to combine with 0.2 g. of iodine would be $18.7 - 0.2 = 18.5$ cc. This means that to obtain an *N/10* solution each 18.5 cc. must be diluted to 20 cc., or that a litre will contain 925 cc. of the solution. To complete the procedure 75 cc. of boiled-out water are introduced into a 1000 cc. measuring flask from a burette and the volume made up to 1000 cc. with the thiosulphate solution. This gives an accurate *N/10* solution which may be tested against 20 cc. of bichromate as

described. The solution will keep for at least several months if preserved in a well stoppered coloured bottle in the dark.

To prepare an $N/100$ solution 20 cc. of the $N/10$ solution are made up to 200 cc. in a measuring flask with distilled water. This weak solution is liable to deteriorate and should be made up fresh every few days. It is important to remember that burettes in which thiosulphate solutions are kept must be cleaned at frequent intervals with a solution of sulphuric acid and bichromate.

No. 5. *Starch solution.*

This is prepared by dissolving about 1 g. of "soluble" starch in 100 cc. distilled water. The solution should be freshly made up though an old solution can be used with success.

No. 6. 20 % *hydrochloric acid solution.*

Prepared by diluting 20 cc. of concentrated hydrochloric (B.P. Sp. gr. 1.16) with distilled water to 100 cc.

STANDARDISING OF HEATING.

This can be very easily accomplished by interposing a small manometer between the gas tap and the burner. A suitable manometer has already been described by Cole [1914], but since the question of temperature is so important, a rough sketch of the most suitable heating and regulating arrangement is given here (Fig. 1). If a suitable manometer is not to hand it is very easy to extemporise one, for all that is required is a T-piece with two pieces of bent glass. These can be connected by means of rubber tubing and are quite as effective as if the whole were in one part. A piece of rubber tubing passing through a screw clip connects the one end of the T-piece with the gas supply, while the other end is connected with the bunsen burner. The burner should have a small chimney to protect the flame. From $\frac{1}{4}$ to $\frac{1}{2}$ inch above this, a ring with ordinary wire gauze is supported, on which the flask is boiled. The gauze must be separated by an interval from the chimney, otherwise the gas will not burn properly. When a suitable flame is determined the position at which the cover of the air inlet cuts the side of the hole in the inner side of the bunsen is marked with a file, as indicated in the diagram, so that the same amount of air can always be admitted by adjusting to this mark. Before proceeding to find a suitable strength of flame, some coloured fluid is put in to the manometer through the open end by means of a capillary pipette; the upper surface of this fluid in both tubes is then indicated by a line on a white card placed behind the manometer or by marks on the two tubes of the manometer itself. When this is done the gas is turned full on, the screw closed down somewhat, and a trial made by placing a conical Erlenmeyer flask containing 23 cc. of sodium sulphate acetic acid solution over the flame. The fluid should be first cooled (or warmed) to 20° C. After a few trials, a flame is easily found which will bring the solution to vigorous boiling in about

1 minute 40 seconds. This is then controlled by taking 20 cc. of above sodium sulphate solution to which are added 3 cc. of the alkaline copper iodine solution and testing as before. In each case, of course, the adjustment of the flame is brought about by loosening or tightening the screw clip. When a strength of flame is found that brings the mixture to *distinct boiling* in about 1 minute 40 seconds to 1 minute 45 seconds, the position of the fluid in both limbs of the manometer is marked (see Fig. 1) either by ink on the glass or on a white card behind, or the difference of level read off and noted. The regulating mechanism is now complete, and whenever it is required at a future time all that is necessary is to adjust the gas pressure by means of the screw until the upper surfaces of the coloured liquid stand at the marks indicated. After some time it will be necessary to add more fluid to compensate for slight losses due to evaporation.

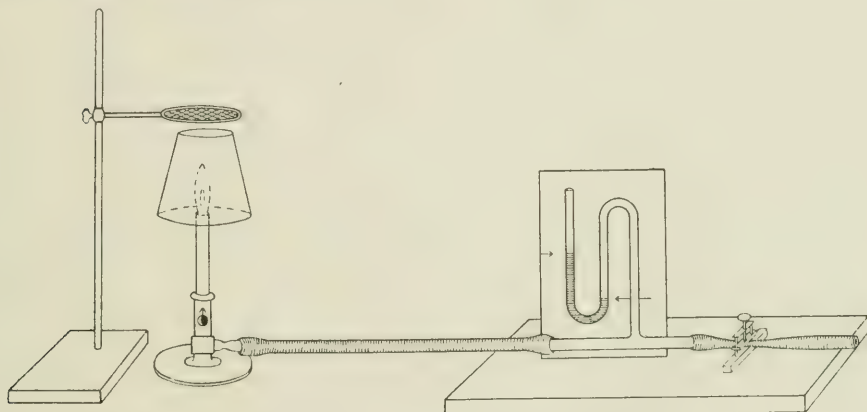


Fig. 1.

The gas pressure in this laboratory varies considerably on some days, while on others it remains practically constant for hours. During careful experiments the manometer must be watched and if a change of pressure occurs the necessary adjustment must be made by means of the screw clip.

I. DETAILS OF METHOD WHEN 1 CC. OF BLOOD IS USED.

Coagulation of protein.

Exactly 26 cc. of acid sodium sulphate solution are run into a small Erlenmeyer flask from a burette. The flask is fitted with a rubber stopper through which passes a glass tube terminating in a capillary point. Blood is drawn from a vein into a test tube containing a small amount of finely ground potassium oxalate and the tube inverted a few times. One cubic centimetre of the blood is measured in a special pipette made by Hawksley "to contain" 1 cc. The blood is allowed to flow out on to the surface of the fluid in the flask. Any blood adhering to the pipette is then washed out by alternately

sucking up and blowing out several times some of the sodium sulphate solution. If the blood is delivered carefully at one side of the flask to begin with, it floats more or less on the surface, leaving the fluid in the other parts of the flask almost clear. If care is taken, the pipette can be washed out with practically blood-free fluid, so that every trace of blood can be delivered. The special stopper with glass tube is now placed firmly in the flask and the mixture heated over a free flame until a bubble or two just appear indicating that the fluid is approaching the boiling point. At this point the flask should be removed. It is then shaken by a circular motion and allowed to stand for one or two minutes. The stopper is then carefully loosened and withdrawn sufficiently to allow the entry of the point of a pipette containing 3 cc. dialysed iron. This iron is added, the flask being shaken gently all the time. It is then shaken two or three times taking care that any drops of fluid in the neighbourhood of the stopper or neck of the flask are washed into the general contents. The flask is then cooled under the tap and the mixture is ready for filtration.

Practically no fluid is lost by coagulating the mixture over the free flame since the small capillary tube does not permit of the escape of much steam while the expanded air makes its exit. Indeed the upper part of the flask remains sufficiently cool to condense steam until the liquid is almost boiling. Even if a little steam does pass into the tube, it condenses there and is sucked back into the flask when air is drawn in on cooling. The large volume of fluid used also minimises any effect of possible loss of traces of moisture by this method.

Filtration.

The contents of the flask are now filtered through a 9 cm. Whatman No. 1 filter paper (W. & R. Balston). This paper is starch-free and I have always found it most suitable for this purpose. Filtration is allowed to proceed until the whole of the liquid has passed through the filter paper; this requires about 6 or 7 minutes. From 23 to 24 cc. of filtrate are obtained. Of this 20 cc. are taken if the blood is from a normal person and likely to contain an average amount of sugar. In the case of diabetics, or if glycosuria is present, 10 cc. filtrate is sufficient. In the latter case the total is made up to 20 cc. by the addition of 10 cc. acid sodium sulphate mixture. These amounts are measured into a small conical Erlenmeyer flask of hard heat-resisting glass. To this exactly 3 cc. of the copper iodide solution is added and the mixture heated.

Heating blood filtrate containing alkaline copper solution.

Over a flame suitably adjusted as already described to bring the 23 cc. of liquid to boiling in about 1 minute 40 seconds the solution is boiled for exactly 6 minutes from the point at which distinct boiling begins. The flask is then removed and either cooled under the tap, or plunged up to its neck into a large volume of cold water in the sink and kept there for about 1 minute. Thorough cooling is essential and must always be carefully carried out.

Treatment of boiled solution.

To the cooled solution are now added carefully with a pipette 10 cc. of 20 % hydrochloric acid and the flask *very gently agitated* at intervals until the effervescence passes over. From this point it is left to stand for about 1 minute or a little more, being shaken with a strong circular motion every few seconds. At the end of this time $N/100$ thiosulphate is run in from a burette and the solution titrated until the yellow colour almost disappears. Two drops of 1 % starch solution are now added and the titration completed. As already stated it is perhaps best not to add the starch at the beginning of titration for there is no doubt that the end point is more distinct if done as indicated here. When carried out in this way, the end point is exceedingly sharp and it is quite impossible for anyone to be more than one drop out. After the titration is finished there is a tendency for the blue colour to return as is usually the case in such solutions, but since this does not happen for several minutes it in no way interferes with the titration.

Calculation of result.

Suppose the blood filtrate as treated above requires 6.69 cc. $N/100$ thiosulphate and 3 cc. of the copper solution alone requires 8.85 cc., then the difference between 8.85 and 6.69 = 2.16 cc. must be due to the iodine absorbed by the cuprous chloride formed. Now from Table I it is seen that 2.16 cc. $N/100$ thiosulphate = 0.6 mg. sugar. Since the amount of filtrate taken corresponds to $\frac{2}{3}$ of 1 cc. of blood, it is obvious that 1 cc. blood contains 0.9 mg. sugar or that 100 cc. of blood contains 0.09 g. of sugar.

Table I.

(For estimation of sugar in 1 cc. blood.)

Showing equivalent of glucose to $N/100$ thiosulphate solution.

Glucose mg.	$N/100$ thiosulphate	Glucose mg.	$N/100$ thiosulphate
	c.c.		c.c.
0.2	0.55	1.2	4.36
0.3	0.95	1.3	4.71
0.4	1.34	1.4	5.07
0.5	1.76	1.5	5.42
0.6	2.16	1.6	5.78
0.7	2.52	1.7	6.13
0.8	2.88	1.8	6.49
0.9	3.27	1.9	6.84
1.0	3.65	2.0	7.20
1.1	4.00		

That the method gives very consistent results is obvious from the following figures obtained on duplicates in six different specimens of blood. Three of

these experiments were carried out on fresh blood, while the others were done on specimens which had stood in the laboratory from 3 to 24 hours.

Fresh bloods			After standing for some hours		
No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
0.096 %	0.102 %	0.089 %	0.059 %	0.048 %	0.030 %
0.098 %	0.108 %	0.086 %	0.066 %	0.046 %	0.030 %

II. METHOD FOR ESTIMATING SUGAR IN 0.2 CC. BLOOD.

The practical advantages of using a small amount of blood have already been discussed and since the manipulation here is quite as simple as is the case when 1 cc. of blood is used, this "micro-method" should prove very useful from the clinical standpoint. This method was partly worked out in 1915, but at the time I was unable to complete it. If the conditions given here are carefully observed, the results obtained are of the same order of accuracy as those in the above method and in spite of the fact that such a small quantity of blood is used no delicate manipulation is required. For this method the following solutions are required. Some of them are similar to those used for the 1 cc. procedure while others are considerably modified.

A. *Solutions for coagulating blood.*

No. 1.

Sodium sulphate	150 g.
Glacial acetic acid ¹	1 cc.
Distilled water to	1000 cc.

No. 2.

Dialysed iron solution "Dialysed iron B.D.H."

B. *Solutions for estimating sugar in filtrate.*

No. 3.

Potassium bicarbonate	12 g.
Potassium carbonate (anhyd.)	8 "
Copper sulphate crystals	0.35 "
Potassium iodate	0.05 "
Potassium iodide	0.5 "
Distilled water to	100 cc.

This is prepared as described under the 1 cc. blood method. It is standardised by taking 2 cc. in 10 cc. acid sodium sulphate solution and adding 2 cc. 75 % HCl. One minute after effervescence is completed and after the mixture has been thoroughly shaken it is titrated as already described with *N*/400 thiosulphate. 2 cc. of this copper solution should require about 11.05 cc. *N*/400 thiosulphate.

¹ The acetic acid should not be added until the solution is required for use.

No. 4.

An $N/400$ sodium thiosulphate solution is prepared from a $N/10$ solution by adding 5 cc. of the latter to some distilled water in a flask and making up to 200 cc.

No. 5.

A solution of "soluble starch" of about 1 % strength.

No. 6.

75 % HCl solution made by diluting 75 cc. concentrated HCl (B.P. Sp. gr. 1.16) to 100 cc. with distilled water.

DETAILS OF METHOD USING 0.2 CC. BLOOD.

The blood is obtained directly from a finger or from the ear. To measure and collect the blood a special pipette¹ is used (Fig. 2), which is shaped so that blood flows in from a slight puncture. If the finger is used it is rubbed with a little ether and thoroughly dried with a cloth. A piece of rubber tubing is then wound round the upper part to cause congestion towards the point and a prick is made with a needle on the back of the finger a little above the root of the nail. The point of the pipette is placed in contact with the side of the drop, when the blood immediately flows in. The pipette is then held horizontally or with the distal end somewhat inclined downwards, so that the passage of the blood into the pipette is helped by gravity. When the blood reaches the mark the pipette is removed and any blood that may remain on the point is carefully wiped away. If the pipette contains too much blood the necessary adjustment is made by gently tapping the point against the thumb nail when a very little blood will escape. If the skin and pipette are clean there is never any difficulty in obtaining the blood, and no particular speed is necessary. Indeed, if the first prick of the needle does not furnish enough blood, the rubber tube may be removed and again wound on and the operation repeated as often as three times or more without any fear of coagulation taking place. Sometimes it may be necessary to squeeze the finger, but since this does not appear to interfere with the result there is no objection to doing so. If the hand is cold it should be placed in hot water for a short time before taking the blood; also, the limb should be held in a hanging position for a few seconds before applying the rubber tube.

The one important point to be observed in using the method is that the *pipette should be kept scrupulously clean*; if any trace of greasy material is present on the inner surface the blood

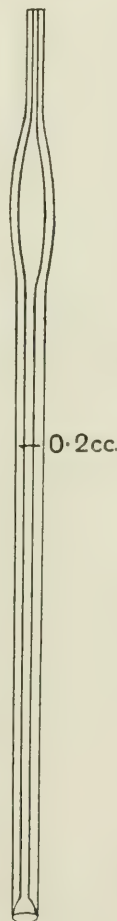


Fig. 2.

¹ This pipette can be obtained from Hawksley & Sons, 357, Oxford Street, London.

will not flow. It is, therefore, most important that after each experiment the pipette should be washed out with a hot mixture of sulphuric acid and potassium bichromate. After thorough treatment with this mixture the pipette is washed out with distilled water followed by alcohol and ether and then dried. Personally, after each experiment I place the pipette in a test tube containing the bichromate mixture and let it stand there until it is again required when it is treated as described. When these precautions are taken there is never any difficulty in getting the blood.

Separation of protein from blood and estimation of sugar.

From a burette 23.8 cc. of acid sodium sulphate solution are introduced into a conical Erlenmeyer flask of heat-resisting glass. To this the 0.2 cc. blood is added and the pipette thoroughly washed out by alternately sucking in and blowing out the mixture. The blood is then heated and treated exactly as in the 1 cc. method, only that 1 cc. of dialysed iron is added instead of 3 cc. This gives a total volume of 25 cc. After cooling and filtering through a 9 cm. Whatman filter paper No. 1, 20 cc. of the filtrate are taken for the estimation of the sugar. In filtering, the process must be allowed to go on until all the material has passed through the filter since only about 21 to 21.5 cc. can be obtained altogether. In diabetic cases 10 cc. of the filtrate are taken and made up to 20 cc. with acid sodium sulphate solution.

To this filtrate are added 2 cc. (carefully measured) of the alkaline copper solution and boiling is carried out exactly as before for 6 minutes using a flame that brings the solution to brisk boiling in about 1 minute 40 seconds. It is then cooled thoroughly, 2 cc. of 75 % HCl added and the further treatment carried out exactly as described for the 1 cc. method except that $N/400$ thiosulphate is used for titration. The end point even with this dilute thiosulphate is perfectly distinct and gives no trouble whatever. The number of cc. of thiosulphate used is then subtracted from the number used by 2 cc. of copper solution alone, and the percentage of sugar in the blood is then calculated from Table II. Thus, if the reading obtained on titration was 8.98 cc. thiosulphate and if 2 cc. copper solution alone gives 11.05 cc., the difference (2.07 cc.) would represent the sugar present. From the table it is seen that 2.07 cc. thiosulphate = 0.18 mg. sugar; therefore $\frac{4}{5}$ (the aliquot part of filtrate taken) of 0.2 cc. blood contains 0.18 mg. glucose. 1 cc. of blood will therefore contain $\frac{1}{1} \times \frac{5}{4} \times \frac{5}{1} \times \frac{0.18}{1}$ mg. glucose = 1.12 mg. = 0.112 %.

This "micro-method" gives excellent results with duplicates as the following numbers show. In blood from the finger the average sugar percentage appears to be somewhat higher than in blood taken from a vein. On an average 0.11 % may be taken to represent the normal. This is probably dependent on the fact that a good deal of the finger blood is capillary in origin and thus differs from the venous sample.

Table II.

(For estimation of sugar in 0.2 cc. blood.)

Showing equivalent of glucose to N/400 sodium thiosulphate solution.

Glucose	N/400 Thiosulphate cc.	Glucose	N/400 Thiosulphate cc.
0.03	0.12	0.22	2.61
0.04	0.25	0.23	2.74
0.05	0.38	0.24	2.86
0.06	0.50	0.25	2.99
0.07	0.62	0.26	3.11
0.08	0.73	0.27	3.24
0.09	0.86	0.28	3.36
0.10	0.99	0.29	3.49
0.11	1.13	0.30	3.61
0.12	1.26	0.31	3.74
0.13	1.39	0.32	3.87
0.14	1.53	0.33	3.99
0.15	1.67	0.34	4.12
0.16	1.80	0.35	4.24
0.17	1.94	0.36	4.37
0.18	2.07	0.37	4.49
0.19	2.22	0.38	4.62
0.20	2.35	0.39	4.74
0.21	2.49	0.40	4.87

Results of duplicate experiments on blood.

No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
0.112 %	0.127 %	0.114 %	0.079 %	0.066 %	0.041 %
0.116 %	0.126 %	0.119 %	0.084 %	0.069 %	0.038 %

The first three samples were obtained from the finger, one sample being taken immediately after the other. The very close results obtained indicate the accuracy of the method. Experiments 4, 5 and 6 were done on three different bloods obtained from a vein; they had stood in the laboratory for varying periods, hence the low results.

The question of the apparent difference in sugar content between capillary and venous blood is at present being investigated; also the effect of different food stuffs and starvation on the blood sugar.

SUMMARY.

A simple method is described by which an accurate estimation of the sugar present in 1 cc. and in 0.2 cc. of blood may be estimated. The method requires careful standardisation of the heating, but otherwise no special care is necessary. With pure glucose solutions theoretical results are obtained and in blood the duplicates always agree within very narrow limits.

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XVII. THE PICRIC ACID METHOD FOR THE ESTIMATION OF SUGAR IN BLOOD AND A COMPARISON OF THIS METHOD WITH THAT OF MACLEAN.

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(Received April 16th, 1919.)

THE colorimetric method for the estimation of sugar in the blood as originally introduced by Lewis and Benedict [1915] has recently been modified by Benedict [1918], and considerably simplified. Addis and Shevky [1918], writing previous to the publication of Benedict's paper, have pointed out that the colour produced by the picric-sugar reaction is not strictly proportional in intensity to the amount of sugar present. Lewis and Benedict in their original paper state that in estimating pure sugar solutions absolutely exact results were obtained, using different amounts of sugar against the same standard, but the work of Addis and Shevky would appear to negative this conclusion. That the latter observers are correct in their statement admits of no doubt and this difference was observed by me before I knew of their paper. Addis and Shevky ascertained that the nearest approach to an exact proportion between intensity of colour produced and amount of sugar present was obtained when an amount of sodium carbonate sufficient to give a concentration of 10 % was present, and the heating was continued for 45 minutes at 100°. Such conditions are not realised in Benedict's modification and in estimations of pure sugar solutions by this method a considerable error is in fact found. In the estimation of sugar in normal bloods, however, the direct error would be of no great magnitude since the sugar content of such bloods approaches closely to that of the standard.

The second possible source of error, the presence of interfering substances in the blood, was also dealt with by Addis and Shevky. They prepared large amounts of filtrates from hyperglycaemic bloods, and, comparing the curves obtained from these under different conditions of alkali concentration and temperature with those obtained from dextrose solutions, came to the conclusion that no interfering substances were present. In these experiments, however, the heating appears to have been prolonged for 45 minutes, no readings having been carried out during the very early stages of the heating.

Benedict replying to the verbal criticism that the picric acid method yields too high results for blood owing to colour production by non-carbohydrate substances gives figures for five samples of dog's blood, tested by the picric acid method before and after treatment of the blood with mercuric nitrate. In two of these samples the apparent sugar content fell after treatment with mercuric nitrate by 19 % and 21 % respectively.

In view of the above possibilities of error in the Benedict method a direct comparison with another method of sugar estimation—that of MacLean [1916, 1919]—appeared advisable. The directions of Benedict were exactly followed, a sugar standard freshly prepared from Kahlbaum's pure glucose being used. On comparing the results obtained in human blood it was found that the Benedict method invariably gave a considerably higher percentage of sugar than that of MacLean. Since Lewis and Benedict [1915] in their original paper noted that haemolysis previous to the addition of the picric solution raised considerably the estimated percentage of sugar, the sugar of the whole blood and of the plasma was estimated in a series of human bloods by both methods with the following results.

No.	Whole blood. Sugar %		Plasma. Sugar %	
	Benedict	MacLean	Benedict	MacLean
(1)	0.114	0.089	0.103	0.099
(2)	0.137	0.104	0.118	0.113
(3)	0.130	0.081	0.112	0.090
(4)	0.176	0.139	0.167	0.147
(5)	0.155	0.092	0.113	0.102
(6)	0.132	0.082	0.114	0.090
(7)	0.157	0.099	0.135	0.110
(8)	0.100	0.067	0.069	0.070

It appears that the sugar content of the whole blood as estimated by the Benedict method is always considerably higher than the result given by the method of MacLean—the excess averaging about 45 % of the sugar present. On the other hand the Benedict results for plasma approximate closely to those obtained by the MacLean method, the excess amounting on an average to about 12 %. While by the MacLean method, the whole blood shows a slightly lower sugar content than the plasma, the Benedict estimation shows a very considerable excess of sugar in the whole blood as compared with the plasma. The difference between the results of the two methods is brought out more strongly if the blood is centrifuged or allowed to sediment in the ice chest and the plasma and corpuscular layers investigated separately.

No.	Plasma. Sugar %		Corpuscles. Sugar %	
	Benedict	MacLean	Benedict	MacLean
(1)	0.108	0.082	0.143	0.064
(2)	0.092	0.079	0.129	0.057
(3)	0.080	0.082	0.113	0.062

Under these conditions while the plasma gives an excess of sugar of 12 % by the Benedict method, the sediment richly laden with corpuscles shows an

average excess of no less than 66 % over the MacLean method. Again, the portion of the blood containing the corpuscles gives a considerably higher figure for sugar than the plasma, while with the method of MacLean, as might be expected from the greater solid content of the corpuscles, less sugar is found in the corpuscular sediment than in the plasma. It should be noted that the figures obtained above do not necessarily represent the original content of sugar in the blood, for several of the specimens had stood in the laboratory for some time before the estimation was carried out.

These results appeared to suggest the presence of an interfering substance in the corpuscles giving a colour reaction with picric acid and raising the supposed sugar content of the whole blood. To some extent they parallel the results obtained by Hunter and Campbell [1917] in creatinine estimations by the Folin method.

A consideration of the very different results obtained by the two methods in whole blood or in blood corpuscles indicates that one of the methods must give entirely fallacious results. If, for purposes of argument, we assume that the error lies in MacLean's method we are forced to accept the extraordinary view that blood corpuscles contain some substance which prevents the sugar present from reducing its equivalent amount of copper when heated in alkaline copper solution. That such a view is untenable is proved by the fact that in the case of blood which has been incubated for a period sufficiently long to allow glycolysis to destroy all the sugar present, any sugar which is added can be recovered quantitatively. If the blood corpuscles contained a substance which interfered with this method, it is difficult to understand why they should not prevent the added sugar from reacting in the normal way. On the other hand a blood so treated gives by Benedict's method a greater percentage of sugar than has been added. Instead of giving values of too low an order, it is highly probable that MacLean's method tends if anything to yield results slightly on the high side, and the different values obtained in the two methods cannot be explained on the supposition that MacLean's method really gives low results.

To prove however that Benedict's results are too high, experiments were carried out to determine whether any direct evidence of the presence of interfering substances could be found in the blood.

If the whole colour reaction was due to sugar, identical readings against a standard sugar solution should be obtained at any stage of the reaction. To test this point samples of the same blood filtrate were heated with a sugar standard for short periods of time, cooled, and rapidly read on the colorimeter. The standards contained as nearly as possible the same amount of blood sugar as the filtrates. Samples of plasma and sedimentated corpuscles were also examined. The concentrations of picric acid and alkali were the same in the sugar standard as in the filtrates to be tested. The results obtained were plotted as curves (Fig. 1). It is obvious from these curves that, while the plasma gives only a slight though distinct variation, the whole blood gives *high initial*

readings falling sharply with prolongation of the heating, and the sedimented corpuscles give even higher readings at the outset with a similar rapid fall. The only possible explanation of this result is the presence of a substance differing from glucose and mainly concentrated in the corpuscles which reacts with picric acid solution more rapidly than sugar, and by its additive effect leads to the ultimate high readings obtained by the Benedict method. This substance is present to a small extent only in the plasma, and consequently estimations carried out on plasma by the Benedict method in normal bloods show only slight excess over those obtained by the method of MacLean. In hyperglycaemic bloods even the results obtained from plasma are likely to be unsatisfactory, owing to the lack of direct proportion between the intensity of colour produced and sugar present under the conditions of the test.

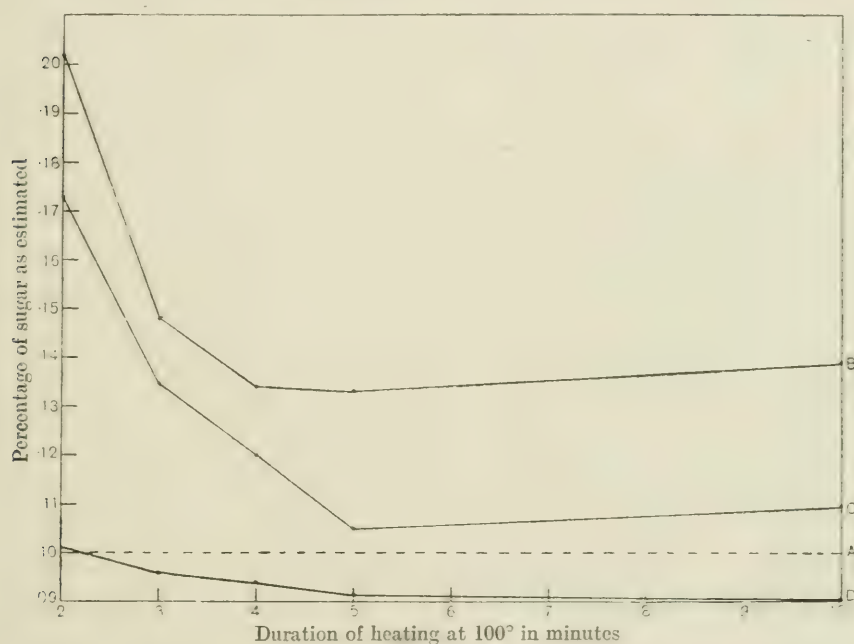


Fig. 1

A glucose standard. B corpuscles C whole blood. D plasma.

CONCLUSIONS.

1. The Benedict method for the estimation of sugar in blood gives results which are too high and shows an average figure about 30 to 50 % in excess of that found by MacLean's method.

2. The high results appear to be chiefly due to the presence of an interfering substance or substances mainly concentrated in the corpuscles but present to some extent in the plasma also; this substance reacts with the picric solution at an early stage of the heating. Creatinine probably plays a large part in this reaction.

3. On account of the influence of this interfering factor the accurate estimation of sugar in whole blood by the picric acid method as described by Benedict is impossible.

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XVIII. ENTEROINTOXICATION—ITS CAUSES AND TREATMENT.

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THE function of the intestinal flora and the relationship of the flora to such of its products as are absorbed by the organism have been matters of some controversy.

Two conflicting views have been held, viz.

- (1) That the intestinal microbes are useful to the organism.
- (2) That the intestinal microbes are harmful to the organism.

The first hypothesis is nothing more than a teleological conception without supporting evidence; the second is based on observations, especially as to the relationship between the intestinal flora and the absence of urinary indican in the breast-fed child.

The contention has hitherto been left undecided owing to the lack of crucial experiments which alone are satisfactory for the elucidation of biological problems.

The present contribution deals shortly with experiments performed with a view to demonstrating that indican and other products in urine are due to the activity of intestinal microbes.

To put this conclusion on an unshakable basis it is necessary to demonstrate that *in vivo* an intestinal flora containing predominantly indole-forming micro-organisms gives rise to indican in the urine, whereas by transforming at will such a flora into one containing in predominance non-indole forming micro-organisms, the indican and other products disappear from the urine.

The first stage in the investigation of the relationship between intestinal flora and urinary poisons is to find a method of transforming the flora. This was done by feeding white rats upon a food diet which included lactose [Distaso and Schiller, 1914]. After feeding for two or three days with this diet the faeces acquire an acid reaction and a yellow colour. The predominant microbe is just the same as in breast-fed infants, viz. the *B. bifidus communis* which never produces indole. We were led to adopt this method starting from the hypothesis that certain enzymes may be absent in adult rats which would result in a sugar passing untouched into the caecum, and so into the large intestine, whereby microbes of what one of us has called the *acetogenous group* (*B. bifidus*, *acidophilus*, etc.) would be able to grow [Distaso, 1911].

Under these conditions just as is the case in experiments *in vitro* the higher the percentage of sugar available for the intestinal microbes the more quickly the acetogenous flora gain the ascendancy, and afterwards they are the only species which survive. We dismissed, therefore, the idea of introducing new microbes into the intestine because in each intestinal flora there are plenty of acid producers and non-indole forming organisms which are always ready to seize the first opportunity for displaying activity. Moreover we found to our astonishment that we were unable to acclimatise strange microbes to the environment of the intestinal flora even though the most favourable conditions were provided [Distaso and Schiller, 1914].

The discovery of this method opens up a wide field from the physiological and pathological standpoint. The rapid sensibility of the intestinal flora to such influence should certainly permit us to solve the question of the relationship between intestinal flora and the urinary poisons, and to acquire a more exact knowledge as regards the activity of the enzymes of the digestive canal in each animal species under normal and pathological conditions and assuredly supplies a more direct and trustworthy method of investigation than any optical method can.

At any rate one problem in connection with the nutrition of the breast-fed child seems to be explained in connection with this discovery. Certainly in this case a sugar is prepared in the breast of the mother, and this is not absorbed by the young organism, but passes untouched into the large intestine. Why the mother prepares in her breast something which is of no nutritive value to the suckling is one of the riddles which it is hard to understand if one carefully avoids teleological explanations.

RELATIONSHIP BETWEEN INDOLE AND MICROBES.

In this connection, the first point which can be demonstrated *in vitro* is that the indole, of which the indican is the oxidation product, is formed by the activity of the microbe alone. In fact if proteins are really split by sterile methods in the presence of intestinal juice, it will be observed that the products of the cleavage reach the tryptophan stage. If the same process is carried out in the presence of microbes, indole will be at once detected. The indole is, therefore, a product of the activity of the microbes, and is not formed by the enzymes of the digestion. This conclusion is supported by Nencki's classical experiments *in vivo*. He found that the products of digestion drawn off through an iliac fistula never contain a trace of indole, whereas it is easy to detect indole in the faeces where we know the microbes display all their activity. Thus *in vitro* as well as *in vivo* it has been demonstrated that indole is formed by the activity of the microbe.

We now proceed to give below some of our experiments which demonstrate that indican disappears from urine if we cause the indole forming flora to disappear, whereas it reappears as soon as the indole forming flora reappears.

We have, moreover, dealt with the question of the production of ethereal sulphates and skatoxyl and we believe there is evidence enough of the mechanism and source of their production.

The feeding experiments were made at intervals over a considerable period, under varying conditions with regard to diet and amount of lactose used. In all cases control feeding experiments were made at the same time as the lactose feeding tests, using groups of from one to three animals as available. The amount of food given (other than lactose) was not weighed, but the animals were of approximately equal size, and were supplied with the food in approximately equal amounts.

Putrefaction of the urine was inhibited by admixture with small amounts of suitable antiseptic.

It was necessary at times to continue the collection for a period of 48 hours or more, although, had the conditions permitted, it would have been more satisfactory to have been able to make analyses upon the freshly collected samples.

ANALYTICAL RESULTS.

Indoxyl. The figures given under this term may be regarded as approximating to the indoxyl present, since the colouring matter was formed in the presence of an oxidising agent, and therefore indigo blue should be the chief product and its amount be approximately in proportion.

10 cc. of urine previously treated with basic lead acetate and filtered, were mixed with an equal volume of pure strong HCl and extracted with 2 cc. of chloroform, adding 1–2 drops of 1 in 10 H_2O_2 .

A second extraction was made where any considerable development of indigo resulted. The intensity of colour was then matched against a standard tint on an arbitrary scale. No great degree of accuracy can be attained by the method and the figures are merely relative, but they serve for purposes of comparison.

AMYL ALCOHOL EXTRACT.

The residual acid liquid was extracted once or twice with small amounts of amyl alcohol, and as with the chloroform extract the coloration obtained was recorded on an arbitrary scale.

To what extent these figures give a measure of the skatoxyl pigment is a matter of controversy; also the tints were not clearly defined and colorimetric readings were more indefinite than with the blue chloroform extracts.

ETHEREAL SULPHATES.

These determinations as well as those of the mineral sulphate were made by the benzidine method as described by Rosenheim and Drummond. The figures are given in terms of milligrams SO_3 per animal per diem. They will

include not only the indoxyl sulphates but also any skatoxyl sulphates, phenol sulphates, etc. It will be found that the few determinations which could be made show some relationship (as is to be anticipated) to the figures for indoxyl.

We give below a few typical experiments for the sake of brevity.

Experiment 1.

Control—Potato
Lactose—Potato + 2.5 g. lactose per animal.
From October 30 to November 16, 1916.

Test			Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO ₃	Ethereal Sulphate mg. SO ₃
1916						
Nov. 3	Control	...	10.5	0.6	6.9	0.6
Nov. 6	„	...	3.5	0.8	7.3	0.3
Nov. 10	„	...	Nil	—	5.0	Nil
Nov. 12	„	...	1.0	—	6.1	Nil
Nov. 16	„	...	Nil	—	2.3	Nil
Nov. 5	Lactose 2.5 g.		Nil	0.1	—	—
Nov. 11	„	...	Nil	—	2.7	Nil
Nov. 16	„	...	Nil	—	1.5	Nil

Remarks. The continued feeding of the control animals with potato only shows a gradual reduction in the figures for indoxyl and also for ethereal sulphates which after two weeks were practically nil.

Although the lactose feeding experiment shows the rapid disappearance of indoxyl and other products within a few days, the experiment is in our opinion inconclusive.

We have been obliged to select another diet which would give a greater and a persistent amount of indican, skatoxyl, ethereal sulphates, etc., in the control, a condition under which alone a crucial experiment would assume its full value.

Experiment 2.

Control—Banana.
Lactose—Banana + 2.5 g. lactose per animal.
From March 14 to March 30, 1917.

Test			Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO ₃	Ethereal Sulphate mg. SO ₃
March 19	Control	...	Traces	2.0	—	—
March 22	„	...	1.5	2.2	0.25	0.1
March 27	„	...	2.4	0.4	0.3	0.1
March 30	„	...	1.9	1.2	0.15	0.05
March 20	Lactose 2.5 g.		0.4	0.2	—	—
March 23	„	...	Nil	0.2	0.15	Nil
March 27	„	...	Nil	1.0	0.1	Nil
March 30	„	...	Nil	0.8	—	—

ENTEROINTOXICATION—ITS CAUSES AND TREATMENT 157

Remarks. In the control feeding the figures for ethereal sulphates (also mineral sulphates) were exceptionally low throughout, but they are capable of comparison.

Lactose feeding gave negative results within a few days for both indoxyl and ethereal sulphates, but the amyl alcohol extract continues to be appreciable in the lactose-fed animals.

Experiment 3.

Control—Expt. 2 Lactose animals now fed with banana.

Lactose—Expt. 2 Control animals now fed with banana + 2.5 g. lactose per animal.

From March 30 to April 23, 1917.

Test			Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO ₃	Ethereal Sulphate mg. SO ₃
April 2	Control	...	Nil	1.6	0.12	Nil
April 4	"	...	1.1	0.5	0.14	Nil
April 6	"	...	7.0	0.7	0.09	0.03
April 7	"	...	2.0	0.5	—	—
April 10	"	...	1.0	1.5	—	—
April 17	"	...	1.2	0.6	—	—
April 19	"	...	3.0	1.0	—	—
April 23	"	...	2.0	1.0	—	—
April 3	Lactose 2.5 g.		Nil	0.3	—	—
April 7	"		Nil	0.3	—	—
April 10	"		Nil	Prac. Nil	—	—
April 13	"		Nil	"	—	—
April 17	Banana only		Nil	"	—	—
April 20	"		0.5	0.5	—	—
April 23	"		0.5	0.5	—	—

Remarks. Feeding reversed. On feeding the original control animals with lactose, negative results for indoxyl were obtained within a few days. The original lactose-fed animals (now banana only) showed a gradual re-appearance of indoxyl.

The same phenomena were to be observed in this experiment as in the previous one; with the appearance of indican, the flora showed chiefly coliform and allied micro-organisms, whereas with its disappearance the acetogenous group partially re-appeared.

We attribute the persistence of the figures for amyl alcohol extract to the fact that it is almost impossible to mix thoroughly the lactose with banana. Further, the transformation of the intestinal flora thereafter was not complete. smears made from films actually showing an appreciable proportion of *B. coliformis*.

Experiment 4.

May 24 to June 8.

Control—Bread for two days previously, then bread and egg.

Lactose—Bread for two days previously, then bread and egg* + lactose: 4 g. to June 2; 5 g. to June 6; 8 g. to June 8 per animal.

	Test		Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO ₃	Ethereal Sulphate mg. SO ₃
May 24	Control	...	14.0	Small amount	—	—
May 27	22.0	Fair amount	—	—
May 29	32.0	1.0	—	—
May 31	16.0	1.6	12.5	1.2
June 2	24.0	1.5	—	—
June 4	28.0	2.0	—	—
June 6	32.0	2.0	—	—
June 8	22.0	2.2	24.1	2.8
May 24	Lactose 4.0 g.		12.0	Small amount	—	—
May 27	..		10.0	Fair amount	—	—
May 29	..		6.0	0.8	—	—
May 31	..		3.0	0.4	4.6	0.25
June 2	..		2.4	1.0	—	—
June 4	..	5.0 g.	< 0.05	0.5	3.4	0.5
June 6	..		Prac. nil	1.0	4.9	0.5
June 8	..		0.8	0.4	4.3	0.3

* This was prepared as follows: an egg was beaten and diluted with 4 times its volume of water. The bread was soaked in this emulsion.

Remarks. The control feeding gave very high figures for indoxyl. The lactose feeding experiment shows a gradual reduction in indoxyl, ultimately to nil, with a large reduction in ethereal sulphates.

The experiment was stopped at this stage, because the rats began to refuse their food and, as will be seen later, this antipathy towards lactose has been an adverse factor to cope with, which has required special conditions of feeding.

Experiment 5.

June 14 to July 16.

Control—Bread and egg; fed on bread for six days previously.

Lactose—Bread and egg + 8 g. lactose per animal up to June 22.

	Test		Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO ₃	Ethereal Sulphate mg. SO ₃
June 14	Control	...	3.0	—	—	—
June 16	13.0	Fair amount	—	—
June 18	30.0	..	—	—
June 20	17.5	—	11.3	0.7
June 22	12.0	1.8	—	—
June 29	Bread only		18.0	Small amount	—	—
June 30	13.0	Fair amount	—	—
July 11	17.5	..	—	—
July 16	16.0	..	—	—
June 14	7.0	—	—	—
June 16	Lactose 8 g.		1.5	Small amount	—	—
June 18	0.2	..	—	—
June 20	0.3	—	0.2	Practically nil
June 22	0.4	Nil	—	—
June 27	Bread only		1.6	Traces	—	—
June 30	1.6	< 0.8	—	—
July 11	4.5	Small amount	—	—
July 16	10.0	Apprec. amount	—	—

Remarks. The control feeding showed high figures for indoxyl and these were still maintained on a reversed diet of bread only. In the lactose feeding a quick reduction was obtained in both ethereal sulphates and indoxyl to practically nil. The indoxyl gradually re-appeared after stopping feeding with lactose, reaching the normal amounts in about three weeks.

Experiments 4 and 5.

Although in these experiments we have been unable to reduce the amounts of indican, amyl alcohol extract and ethereal sulphate to nil, this partial failure is, in our opinion, due to certain adverse circumstances, *viz.*

- (1) The diet was really very severe.
- (2) We have been obliged to use old animals which owing to long experience had become unsuitable for use.
- (3) The rats have a distaste for lactose.

In order to avoid a condition of starvation in this and in other experiments they were discontinued whenever these adverse factors were becoming acute.

Experiment 6.

July 23 to August 18.

Control—Fed on bread three days previously, then bread and egg.

Lactose—Fed on bread three days previously, then bread and egg to July 26, then 8 g. lactose, then 5 g., then 10 g. lactose per animal.

Test	Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO ₃	Ethereal Sulphate mg. SO ₃
July 23 Control ...	28.0	Apprec. amount	—	—
July 25 „ ...	20.0	1.5	13.4	1.0
July 28 „ ...	19.0	1.7	—	—
July 31 „ ...	33.0	3.2	—	—
Aug. 3 „ ...	16.0	1.1	14.3	0.7
Aug. 6 „ ...	27.0	1.5	—	—
Aug. 10 „ ...Large amount		1.1	—	—
Aug. 13 „ ...	21.0	1.8	16.4	1.1
Aug. 15 „ ...	10.0	Small amount	—	—
Aug. 18 „ ...	9.0	Fair amount	—	—
July 23 Bread ...	32.0	Fair amount	—	—
July 25 Bread and egg	19.0	1.9	13.2	1.0
July 28 Bread and egg				
+ Lactose 8 g.	< 1.0	0.3	—	—
Aug. 1 „	0.4	0.2	—	—
Aug. 3 „ 5 g.	0.6	0.5	6.8	Nil
Aug. 6 „	0.4	Practically nil	—	—
Aug. 10 „	Trace only			
	< 0.05	0.2	—	—
Aug. 13 „ 10 g	0.3	Practically nil	0.2	Nil
Aug. 15 „	2.0	Traces	—	—
Aug. 18 „	4.2	Apprec. amount	—	—
		< control		

Remarks. The control feeding showed high figures for indoxyl which were reduced towards the end of the period. With lactose feeding the amounts of both indoxyl and ethereal sulphates were quickly reduced to almost nil and finally also the mineral sulphates, but afterwards the indoxyl began to reappear.

This is an illustrative experiment in which the results obtained may appear to indicate a failure on our part to produce systematically and always the disappearance of urinary poisons. But the flora in this case suggests the explanation. Here it is to be noted that there is an enormous fall in urinary poisons after three days of lactose feeding, but at the same time the flora was far from showing the homogeneity seen in other cases. This condition had continued for 13 days, when we decided to increase the amount of lactose. Now the rats took little food, it being almost entirely rejected—a condition of starvation in which all the classical symptoms ensued and which led to a definite increase in the urinary poisons. The flora on the other hand again returned to the condition of the control flora, viz. predominance of *B. coliformis*.

Experiment 7.

September 17 to October 7, 1917.

Control—Bread and egg.

Lactose—Bread and egg + 8 g. lactose per animal.

	Test	Indoxyl	Amyl Alcohol Extract
Sept. 17	Control Bread ...	6.0	1.2
Sept. 19	„ Bread + egg	6.5	1.9
Sept. 21	„ „	8.0	2.6
Sept. 24	„ „	12.0	2.3
Sept. 26	„ „	10.0	1.8
Sept. 29	„ „	7.2	1.3
Oct. 1	„ „	14.5	1.2
Oct. 4	„ „	13.0	1.3
Oct. 7	„ „	13.0	0.5
Sept. 17	„ „	5.5	1.4
Sept. 19	Lactose 8 g. ...	2.0	0.5
Sept. 21	„ „	1.0	0.3
Sept. 24	„ „	2.2	0.4
Sept. 26	„ „	1.6	0.4
Sept. 29	„ „	1.2	0.6
Oct. 1	„ „	1.1	0.7
Oct. 4	„ „	1.5	1.0
Oct. 7	„ „	0.5	0.5

Remarks. Old rats used many times. The control feeding gave indoxyl in fair amount, but less than in earlier experiments. With the lactose feeding a slow gradual reduction in amount of indoxyl is to be noted. A certain amount of indican, although small, persisted to the end of the experiment, and an observation of interest is that the flora always contained a certain number of Gram-negative bacilli (*B. coliformis*).

ENTEROINTOXICATION—ITS CAUSES AND TREATMENT 161

As previously stated, the rats were very fastidious in choosing their food, selecting the crumbs and rejecting lactose so far as possible, and of course without lactose introduced into the intestine the experiment will fail.

It is very difficult to secure a thorough admixture of lactose with bread and therefore for later experiments we chose cooked potatoes to which an emulsion of egg was added.

Experiment 8.

Control—Potato and egg.

Lactose—Potato and egg + 8 g. lactose per animal.

Nov. 14 to Nov. 27, 1917.

	Test			Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO ₃	Ethereal Sulphate mg. SO ₃
Nov. 14	Control	8.5	—	—	—
Nov. 20	„	8.5	Trace only	—	—
Nov. 24	„	5.0	Practically nil	—	—
Nov. 27	„	8.0	„	10.4	0.6
Nov. 14	Before Lactose	8.4	—	—	—
Nov. 20	+ Lactose 8 g.	0.2	—	—	—
Nov. 24	„	Nil	0.3	—	—
Nov. 27	„	Nil	Practically nil	7.3	Nil

Remarks. The control feeding showed indoxyl and ethereal sulphates in fair amount, less than in the earlier feeding with bread and egg, but the amounts were persistent throughout the experiment. With the lactose feeding a rapid elimination of indoxyl occurred with the practical elimination of ethereal sulphates within ten days.

Experiment 9.

Control—Potato and egg.

Lactose—Potato and egg + 8 g. lactose per animal.

December 18, 1917, to January 7, 1918.

	Test			Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO ₃	Ethereal Sulphate mg. SO ₃
Dec. 20	Control	8.5	Practically nil	—	—
Dec. 22	„	5.0	0.6	—	—
Dec. 24	„	4.5	0.7	8.4	0.9
Dec. 25	„	Fair amount	Small amount	—	—
Dec. 31	„	4.5	„	—	—
Jan. 2	„	7.4	„	—	—
Jan. 4	„	7.8	1.3	18.0	0.8
Jan. 7	„	8.0	Nil	14.5	1.9
Dec. 20	Lactose 8 g.	0.2	Practically nil	—	—
Dec. 22	„	Nil	Nil	—	—
Dec. 24	„	Nil	Nil	—	—
Dec. 28	„	Nil	Nil	—	—
Dec. 31	„	Nil	Nil	—	—
Jan. 2	„	Nil	Nil	—	—
Jan. 4	„	Nil	Nil	8.3	Nil
Jan. 7	„	Nil	Nil	12.3	Nil

Remarks. The control feeding results were similar to those in Experiment 8, whilst the lactose experiment also gave results practically identical with the previous Experiment 8. With this diet we have, therefore, reached the ideal experimental conditions for proving our assertion and in this and the following experiment it will be seen that with these conditions realised the experiments will give consistently the same results.

Experiment 10.

Control—Potato and egg.

Lactose—Potato and egg + 8 g. lactose* per animal.

March 12 to 26, 1918.

	Test		Indoxyl	Amyl Alcohol Extract	Mineral Sulphate mg. SO_3	Ethereal Sulphate mg. SO_3
March 18	Control	3.4	1.2	—	—
March 19	8.4	2.0	24.0	2.2
*March 20	5.4	Small amount	—	—
*March 22	9.6	..	15.9	2.45
*March 23	12.8	..	—	—
March 25	19.0	..	16.3	2.0
March 26	17.0	Yellowish	—	—
March 18	Lactose 8 g.	...	Nil	Nil	—	—
March 19	Nil	Nil	—	—
March 20	Nil	Nil	—	—
March 22	Nil	Nil	—	—
March 23	Faint trace	—	—	—
			< 0.1	Practically nil		
March 25	Nil	Nil	7.7	Nil
March 26	Nil	Nil	13.2	Nil

* Since under the conditions of the experiments some direct admixture of lactose with the urine was unavoidable, an amount of lactose approximately equal to that given in the food was in these control tests added to the containing vessels, giving a direct admixture of the urine with lactose with no marked fluctuations in the results obtained.

Remarks. In the control feeding the amounts of indoxyl and ethereal sulphates reached a higher figure than with previous experiments. The lactose experiment showed a quick elimination of indoxyl and also of ethereal sulphates.

CONCLUSIONS.

We have been able to show that the presence of indoxyl, skatoxyl and ethereal sulphates in the urine, corresponds with a flora composed of microbes which *in vitro* give indole and skatole.

On the other hand if such a flora be transformed into one which does not produce either indole or skatole, then indoxyl, skatoxyl and ethereal sulphates are absent from the urine.

The group of microbes which produces these poisons is that of *B. coli-formis* which play a great rôle in many intestinal disorders and states of ill-health, and to produce disintoxication the intestinal flora must be transformed so as to consist of non-indole producers.

It is interesting to note that throughout our experiments the total mineral sulphates are lower in the non-intoxicated animals, and this observation should open up an experimental field of considerable importance in relation to the metabolism of the mineral constituents of the animal body.

We admit that the amount of mineral matter in the food used will influence the excretion of the mineral sulphates, but nevertheless our experiments show throughout a marked decrease of the total mineral sulphates with lactose feeding.

This effect we attribute to the fact that in intestinal stasis and in cases where intestinal fermentation is abnormal, the food is far more broken down by the action of the microbes and therefore more absorption of the mineral products takes place, with greater excretion in the urine.

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XIX. THE ACTION OF ULTRA-VIOLET RAYS ON THE ACCESSORY FOOD FACTORS.

BY SYLVESTER SOLOMON ZILVA.

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(Received April 28th, 1919.)

IN spite of the various attempts to isolate and identify the accessory food factors their chemical identity still remains unsolved. As far as the antineuritic and the antiscorbutic factors are concerned it is possible now by means of chemical fractionation to obtain residues which are very active. These preparations contain comparatively little impurity judging from the small quantities required to produce a therapeutic effect. Although the chemical aim has not been achieved the possibility of employing such active fractions has been found useful in furthering research on the accessory factors in the domain of nutrition.

As the chemical identification of these principles has not so far been successful by means of fractionating methods it becomes advisable to study their properties, because it contributes to the knowledge of the nature of these unknown substances. During the various investigations carried out on the accessory factors many of their properties have already been revealed. So far, however, the action of light on them has not been studied. This investigation is devoted to the study of ultra-violet rays on the accessory factors. These rays are well known to produce pronounced chemical changes in many compounds and also to influence the physiological activity of various biological principles, and it was therefore of interest to find out the effect on the accessory factors after an exposure to ultra-violet rays. The action of the light on the antiscorbutic, antineuritic and the fat-soluble *A* factor was investigated. As these factors cannot be obtained in pure form they could only be exposed to the ultra-violet rays as present in active substances. The antiscorbutic was used in the form of lemon juice from which the citric and other acids have been removed as described by Harden and Zilva [1918]. Autolysed yeast was used for the antineuritic and butter was employed as the source of the fat-soluble *A*.

EXPERIMENTAL.

The Antiscorbutic Factor.

In using the treated lemon juice for the antiscorbutic factor it was brought to a definite H-ion concentration in order to equalise the conditions. This was done colorimetrically by the addition of *N*/10 HCl to the juice and matched against a standard solution of glycine and *N*/10 HCl estimated by the gas

chain method to be P_H 2.34. The H-ion concentration was adjusted daily before the liquid was exposed to the ultra-violet light. The solution was introduced in a quartz tube of 1 inch diameter which was revolved by means of a water motor. As a source of ultra-violet rays the quartz mercury vapour lamp was used and placed about 6 inches from the revolving tube. The solution was exposed to the light for 8 hours. Three sets of animals were employed in this experiment. One set received the treated lemon juice without having its reaction adjusted, the other set received the treated lemon juice with its reaction adjusted as described above and the third set received the treated lemon juice brought to P_H 2.34 and exposed for 6 hours to the ultra-violet rays. Each set had four animals which received 0.5 cc., 1.5 cc., 2.5 cc. and 5 cc. of the respective solutions. Fig. 1 represents the weight curves of these animals.

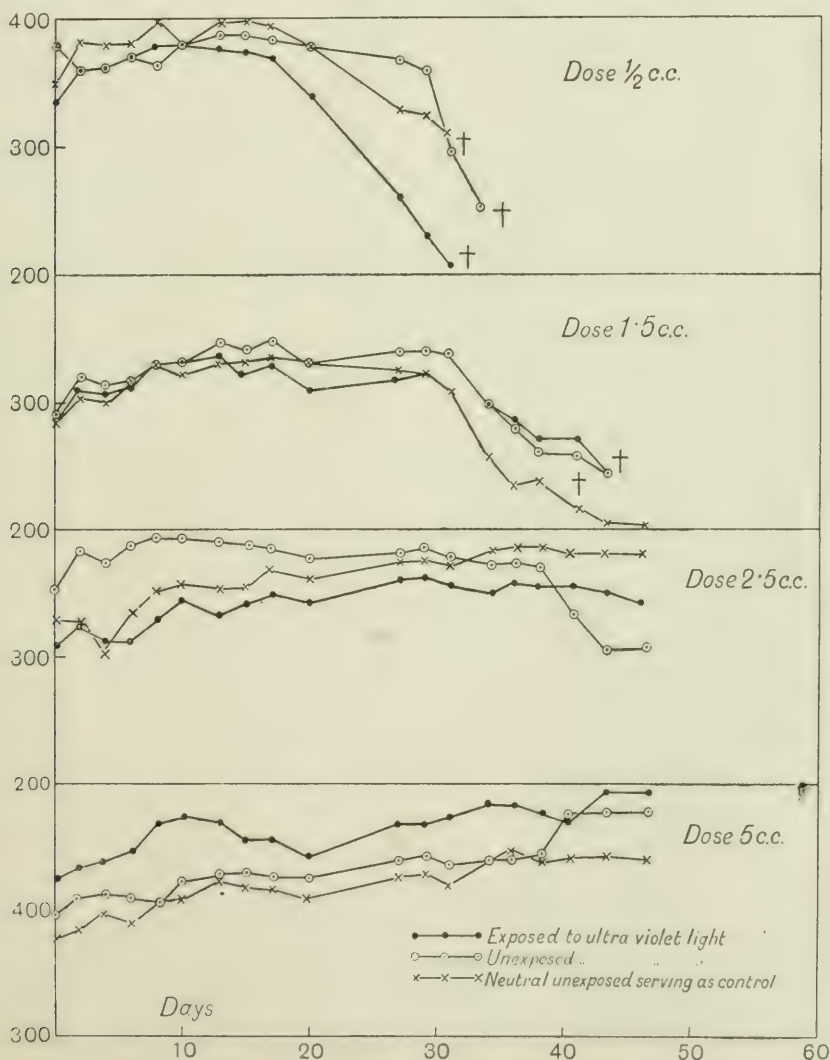


Fig. 1

The animals in all the sets which received 0.5 cc. of the solution succumbed to scurvy within about 30–35 days. Of the animals receiving 1.5 cc. of the various solutions two died of scurvy within 42 days, and the third one receiving the treated lemon juice as a control was decreasing rapidly in weight when it was chloroformed on the 46th day. The post mortem revealed very marked scurvy. The guinea pigs which received 2.5 cc. of the solution all survived the duration of the experiment, namely 46 days. These animals were chloroformed and at the post mortem examination no signs of scurvy could be detected. The dose of 5 cc. also warded off scurvy entirely in all cases. These animals were growing when they were chloroformed after they had been under experiment for 46 days. They were found in good condition and no traces of scurvy could be detected.

One is bound to conclude from the above experiment that the exposure to the ultra-violet rays did not destroy or even impair the antiscorbutic activity of the treated lemon juice at the P_H 2.34.

An attempt was then made to ascertain whether the action of ultra-violet rays has any effect on the treated lemon juice in neutral condition. Lemon juice treated by the Harden and Zilva method is neutral or slightly acid to litmus; this reaction depends on how thoroughly the organic acids have been removed from the lemon juice. By taking the necessary precautions it is possible to prepare it always so that it reacts neutral to litmus. Such neutral preparations of lemon juice were used in this experiment. One group of guinea pigs received the exposed treated lemon juice; to the other group the unexposed treated lemon juice of the same batch was administered. As in the previous experiment doses of 0.5 cc., 1.5 cc., 2.5 cc. and 5 cc. were given. In this case too both animals receiving 0.5 cc. declined and died of scurvy within about a month, thus showing 0.5 cc. of the treated lemon juice to be a dose insufficient to delay or prevent the onset of scurvy in guinea-pigs. The dose of 1.5 cc. in both cases delayed but did not prevent the onset of scurvy. The animal receiving the solution exposed to the ultra-violet rays was chloroformed and found to be suffering from acute scurvy. The guinea pig which received the control solution died of scurvy after 33 days. 2.5 cc. and 5 cc. of both solutions was sufficient to prevent scurvy. The weight curves of the above animals (Fig. 2) show that no great differentiation can be established in the growth of the animals receiving similar doses of the respective solutions. It is therefore evident that the action of the ultra-violet rays produced no marked deterioration of the antiscorbutic potency of the neutral treated lemon juice.

The Antineuritic Factor.

In studying the influence of ultra-violet rays on the antineuritic accessory factor pigeons and rats were employed. A pigeon was kept on a diet of polished rice until it developed polyneuritis; a dose of the exposed autolysed yeast was then administered in order to see whether it had a curative effect. The

autolysed yeast was exposed to ultra-violet light in a similar manner to the lemon juice. The following protocol describes the experiment in detail.

Pigeon *H* showed decided symptoms of polyneuritis. Its head was bent well forward. The animal was unable to walk and could not stand firmly on its legs. 3 cc. of the exposed autolysed juice was administered per

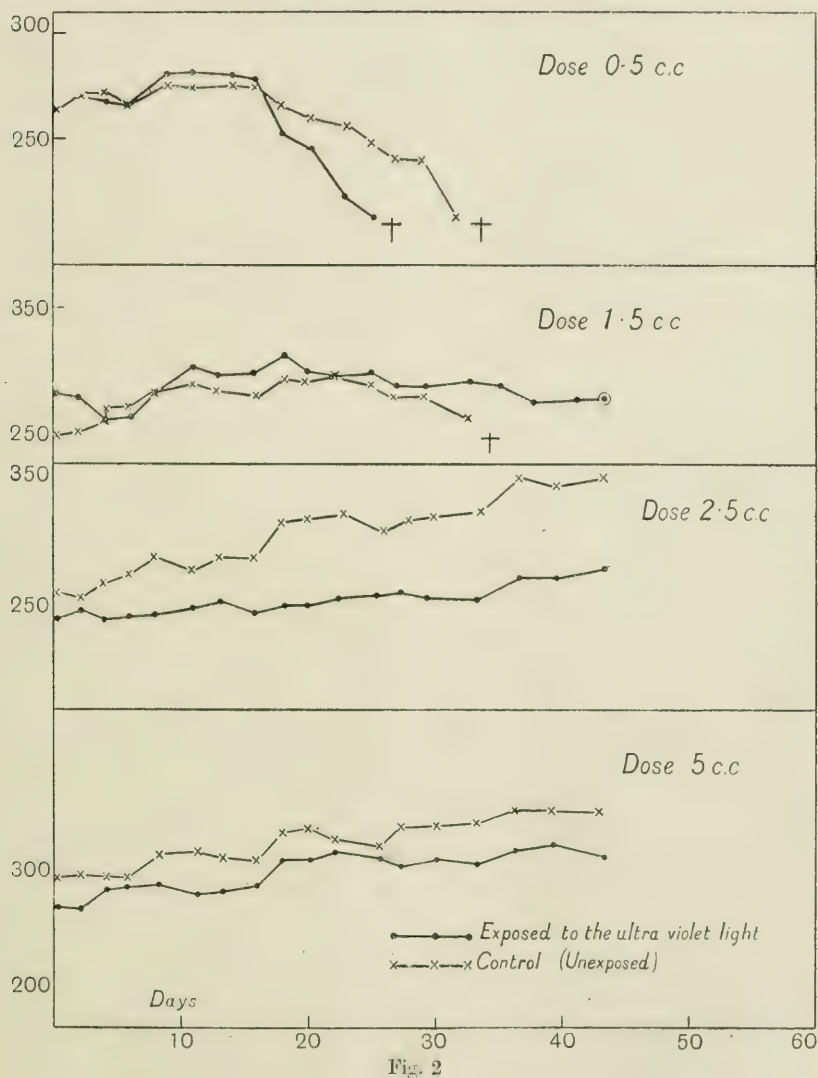


Fig. 2

os at 2 p.m. and another 2 cc. at 4.30 p.m. At 6 p.m. the same afternoon an improvement was observed. The following day the animal was normal. It was kept on a rice diet and after 10 days it developed another attack of polyneuritis. 2 cc. only were administered this time. The animal nevertheless improved and kept up its condition for 6 days.

The above experiment shows that the exposure to the ultra-violet rays did not entirely destroy the antineuritic activity of the autolysed yeast. It was desirable to ascertain whether the antineuritic activity was at all impaired by the exposure to the light. Rats were found convenient animals for that purpose. These animals if kept on a diet free from the antineuritic do not grow and eventually die after about 6 weeks. If, however, the antineuritic is supplied after the deficiency has been enforced for several weeks normal growth is immediately resumed.

In this experiment three rats were employed. Their diet was made up as follows:

Basal mixture	10 g.
Antiscorbutic equivalent to lemon juice	5 cc.
Centrifuged butter-fat	1.8 g.
Olive oil	1.6 „

The basal mixture was made up of 75 % starch, 20 % caseinogen and 5 % salts.

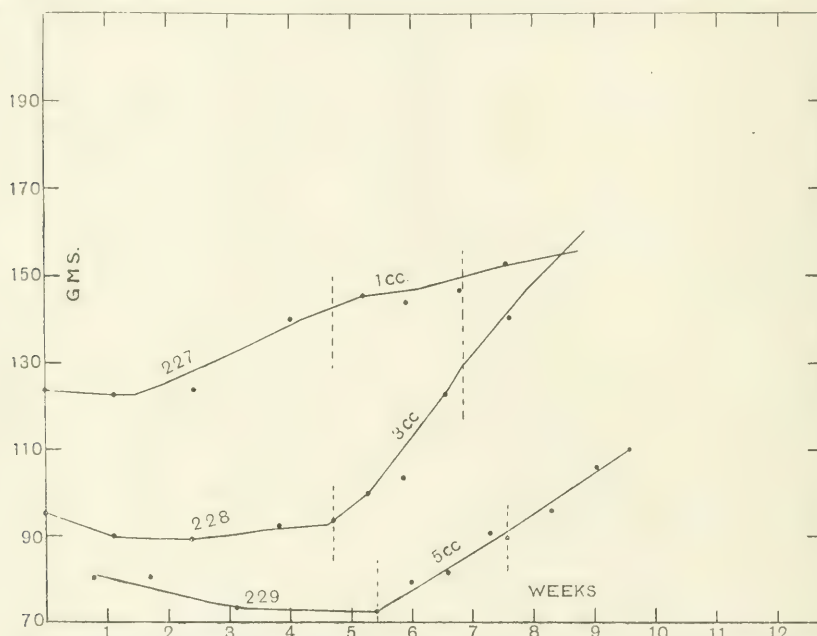


Fig. 3. The first perpendicular dotted line marks the time at which the administration of the exposed juice was commenced and the second line that of the unexposed juice of the same batch.

As will be seen from Fig. 3, two of the rats (Nos. 228 and 229) showed no growth for $4\frac{1}{2}$ weeks; the third animal (No. 227) showed little growth, much below the normal. After $4\frac{1}{2}$ weeks No. 227 received in its daily ration 1 cc., No. 228 3 cc. and No. 229 5 cc. of autolysed yeast juice exposed to ultra-violet rays. All the three animals commenced growing. The improvement

was least marked in rat No. 227 which received only 1 cc. of juice, the dose being evidently insufficient. After 15 days the exposed autolysed juice was replaced by unexposed autolysed juice which belonged to the same batch, and as the curves show the growth in all the three cases proceeded at the same rate as before. This experiment shows that the exposure of autolysed yeast to ultra-violet rays for 6 hours neither destroys nor vitiates to any marked degree the antineuritic activity of the juice.

The Fat-soluble A Accessory.

The influence of the ultra-violet rays on the fat-soluble *A* factor was studied next. As butter was used for the source of this accessory factor the technique had to be somewhat modified. The butter was spread in a thin layer over a glass plate which was placed in a horizontal position about 6–8 inches from the lamp. The butter was turned over several times during the exposure. The part of the plate nearest the lamp became slightly warm from the heat of the lamp but it did not reach a higher temperature than 30°. It soon became apparent that the butter was greatly altered by exposure to the ultra-violet light. It became entirely bleached, acquired an odour like that of tallow, and was unpalatable and unfit for consumption. Several preliminary experiments showed that exposure to the ultra-violet rays destroyed the fat-soluble *A* in the butter and this was confirmed on repetition. The following experiments demonstrate it. The mode of procedure adopted was the same as that employed by Drummond [1919]. Three rats were placed on a diet free from the fat-soluble *A* factor, which was made up as follows:

Basal mixture	12 g.
Antiscorbutic equivalent to lemon juice	5 cc.
Autolysed yeast juice	5 „
Olive oil	0.75 cc.

Precautions were taken to destroy any traces of the fat-soluble *A* that might be present in the basal mixture by heating the starch and the caseinogen for 3 hours at 100° C., since it has been shown by Steenbock, Boutwell and Kent [1918] and Drummond [1919] that this factor is thermolabile. The animals were kept on this diet for 18 days after which time 2 g. of the exposed butter was added to the above daily ration. This diet was continued for 25 days, when the exposed butter in the diet was replaced for two rats by ordinary butter kept for 6 hours at 35°. The butter was kept at this temperature in order to demonstrate that the slight heating effect of the lamp, which, as already mentioned, never increased the temperature to more than 30° at the nearest point, was not responsible for the destruction of the factor. On examining Fig. 4 it is seen that during the first period when the rats were receiving the diet lacking the fat-soluble *A* factor two of the animals did not grow at all, the third animal did grow, but at a rate much below the normal. The addition of the exposed butter made no difference to the growth of either

of the animals. One of the rats left on that diet developed xerophthalmia and died after about 6 weeks. When the exposed butter was replaced by unexposed butter kept for 6 hours at 35° in the diet of the remaining two rats they at once commenced growing, thus showing that the fat-soluble *A* was supplied by the addition of the unexposed but not by the exposed butter. The fat-soluble *A* factor in the butter was therefore inactivated by exposure to the ultra-violet rays.

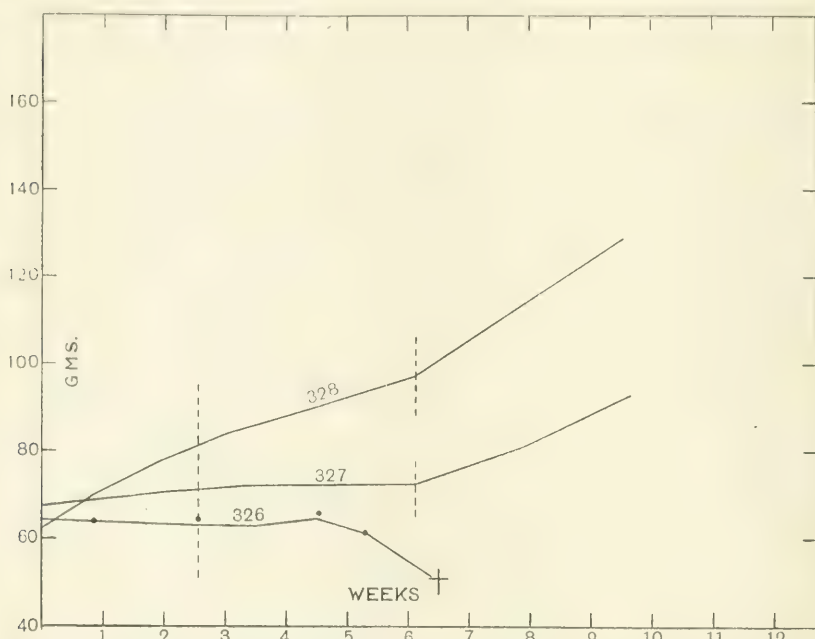


Fig. 4. The first perpendicular dotted line marks the time at which the administration of the exposed butter commenced and the second line that of the unexposed butter.

On exposing the butter to the ultra-violet rays by means of the technique described above two other factors are introduced which might be suspected to bring about the inactivation of the fat-soluble *A* factor, namely, (a) temperature and (b) ozone, which, as is well known, is produced by the action of ultra-violet light on the atmospheric oxygen. That the slight elevation of the temperature of the butter is not responsible for the inactivation is seen from the above experiment, as the addition to the diet of butter not exposed to the ultra-violet light but kept for 6 hours at 35° at once induces growth. The part played by the ozone in the inactivation of the factor, as well as the chemical changes produced by the ultra-violet light in the fat, are now being studied.

In this connection it is of interest to add that it has been previously pointed out by many investigators that the sterilisation of milk by means of ultra-violet rays imparts to the milk a peculiar taste. This is no doubt due to the alteration in the butter fat brought about by the exposure. In view of

the above observations one would also expect that the action of ultra-violet light on milk might greatly impair the activity of the fat-soluble factor in it and thus diminish its nutritive value, especially when used for the purpose of infant feeding.

SUMMARY.

1. The exposure of treated lemon juice to ultra-violet rays in neutral condition and at a H-ion concentration of $P_{H} 2.34$ for 8 hours does not influence its antiscorbutic activity.
2. The exposure of autolysed yeast juice for the same length of time does not impair its antineuritic potency.
3. Butter exposed for 8 hours to ultra-violet light undergoes a very noticeable change and the fat-soluble *A* factor in it becomes inactivated.

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XX. THE INFLUENCE OF DEFICIENT NUTRITION ON THE PRODUCTION OF AGGLUTININS, COMPLEMENT AND AMBOCEPTOR.

By SYLVESTER SOLOMON ZILVA.

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(Received May 2nd, 1919.)

At least two diseases, scurvy and beri-beri, can definitely be traced to a prolonged consumption of a faulty diet. Both experimental and clinical data fully support this view which is now accepted by the majority of scientific workers and clinicians. Other diseases designated as "deficiency diseases," like pellagra, rickets, sprue, etc., are also attributed by some to a dietary deficiency, but the scientific evidence available at present is not sufficient to warrant any definite conclusion as regards the etiology of these diseases.

As is well known scurvy and beri-beri are caused by the absence from the diet of two principles of unknown chemical constitution. By depriving the diet of certain animals of these principles pathological conditions closely resembling scurvy and beri-beri can be induced. Lately Osborne and Mendel [1912, 1] and McCollum and Davis [1913] have demonstrated the necessity of a third accessory food factor provisionally named "the fat-soluble *A* factor" for the growth and existence of the rat. This has been confirmed by other workers. The effect of this deficiency on the human subject is still uncertain, but experimental evidence is gradually accumulating which promises to reveal it.

Besides the above principles of unknown chemical constitution it has been demonstrated experimentally on various kinds of animals that the removal of some chemically known compounds and elements from the diet restricts growth in the young and makes the food inadequate for sustenance in general. These products are of inorganic and organic nature.

The indispensability of the inorganic constituents of the diet was disclosed by the early researches in nutrition. The deficiency of certain elements such as calcium or phosphorus makes itself manifest very soon in growing animals. The presence of other inorganic elements in the food also exercises a very important physiological function.

The organic portion of the diet is limited by the character of the proteins as regards their amino-acid content. Willcocks and Hopkins [1906] and

Osborne and Mendel [1912, 1, 2, 3 and 1913] have shown that certain amino-acids are indispensable for existence and cannot be synthesised by the animal organism. Consequently these must be supplied in sufficiency through the medium of the consumed food in order to ensure the well-being of the animal.

When, therefore, any of the accessory food factors or the indispensable organic or inorganic constituents are eliminated from the diet, the animals cease to grow and eventually die. In the case of deficiencies in the antineuritic and antiscorbutic factors, as already pointed out, the animals succumb to clinically recognised diseases. The pathology of the other deficiencies is not so well defined. In all cases, however, where the diet is deprived partially or completely of an essential constituent the animals under experiment soon manifest the deficiency by a retarded rate or absolute cessation of growth, by restricted propagation and by a general unhealthy appearance, so that if any vital principle of the food is withheld for a prolonged period organic changes ensue which are very marked. But besides the radical changes produced by deficient nutrition already recorded it is quite possible that decided modifications may take place in the body tissues and fluids which are not discernible macroscopically or microscopically, but which may nevertheless restrict physiologically the functions of the organism. In this connection arises the interesting problem whether the resistance to disease of the animal is in any way influenced by deficient nutrition.

There is a general belief that underfed individuals are more susceptible to infection than well fed persons and that when the former contract a disease they show less resistance and are more prone to succumb to it. But terms like "malnutrition" or "underfed" are vague expressions and lack scientific definition. There is also no definite scientific evidence even of a general character to support this belief in spite of its probability. If, however, there is a connection between imperfect nutrition and susceptibility to disease the subject becomes one of the utmost theoretical and practical importance.

Even with our present knowledge of the various foodstuffs as regards their chemical constitution and content of the accessory food factors it becomes evident on reflection that the food of some people living under modern conditions may not contain an evenly distributed and adequate amount of all the vital factors which are necessary for normal existence. It is true that, excluding abnormal conditions like war or famine, the probability of a prolonged *total* deficiency of one or more essential principles from the diet is remote, but a prolonged partial deficiency or a rigorous deficiency lasting a short period is quite possible. The effect of such an irregularly balanced diet need not necessarily manifest itself by such drastic constitutional changes as those produced by the absolute deprivation of any essential principle for a long period, but it is only reasonable to suppose that certain systemic modifications may take place. This conjecture finds some support in the observations made by Zilva and Wells [1919] who found decided histological changes in the teeth of guinea-pigs kept on a scorbutic diet at a stage when the animal

was growing normally and was apparently in normal health. One may similarly imagine that the body cells and fluids may be altered at an early stage by deficient nutrition. It becomes necessary therefore to ascertain in the first place whether the immunity of an animal is affected by a rigorous and prolonged dietetic deficiency.

Immunity is a complicated biological phenomenon and our present knowledge of the subject is a collection of certain facts supposed to be connected with it, which are, however, insufficient to explain the actual mechanism involved. Immunity, therefore, is not very amenable to quantitative estimation. That it is a variable function is evident from empirical observations. Certain phenomena, however, like phagocytosis, complement fixation, agglutination, etc., connected with immunity can be estimated quantitatively for comparative purposes, and it is therefore possible to investigate the influence of diet on such phenomena.

So far there seems to be no record of any experimental work on this subject. It has been asserted by some observers that vegetarian races are less resistant to disease than races which eat meat. Even if that assertion be correct other differential conditions under which these races live would prevent one from ascribing the lower resistance of vegetarian races to their diet. Also the resistance of carnivorous animals towards certain diseases to which herbivorous animals are subject and *vice versa* cannot be entirely explained by the differential diet owing to the number of other factors concerned.

In studying the influence of diet on immunity one has to deal with a multitude of phenomena which offer a great variety of problems to be settled. As already mentioned a diet may be deficient by lacking one of the vital chemically known or chemically unknown principles, but the deficiency may also be of a multiple character. The conceivable changes in the factors connected with immunity which may be produced in the animal organism by any deficient diet are also numerous. The problem, therefore, is very complex and its investigation difficult.

In dealing with immunity one has to consider natural immunity and acquired immunity. The former is the inherent capacity of the animal to resist infection and the latter the immunity resulting from accidental or artificial infection previously sustained. The experimental study of natural immunity would have to be carried out by injecting standardised doses of a suitable lethal organism into animals fed on the various diets to be investigated and recording the relative mortality and time taken by the animals to succumb. In order to draw definite conclusions such experiments would require a great number of animals in each set. The evident drawback of such a plan of investigation is the amount of labour necessary for the preparation of purified diets for the great number of animals a systematic inquiry of such a nature would require. The task becomes almost impossible for a single experimenter to carry out. In investigating acquired immunity one is again confronted with the difficulty of choosing from the great number of factors

which undergo a change during artificial or accidental immunisation those most suitable for study.

In this investigation the study of the influence of deficient diets on the amboceptor and agglutinin formation and the complement content has been undertaken. Owing to the complexity of the subject the experiments carried out can only be considered of a more or less preliminary character. The results obtained although of an unexpected nature are nevertheless of general interest and suggest the necessity of further investigation.

The influence of the following dietetic deficiencies on the production of the above-mentioned antibodies was studied.

(a) The deficiency of each of the following elements Ca, Fe, K, Cl, P, and Na in the inorganic content of the diet.

(b) The deficiency of certain amino-acids.

(c) The deficiency of the antiscorbutic, antineuritic, and the fat-soluble A accessory factors.

The influence of a quantitatively restricted diet on the complement content was also investigated.

EXPERIMENTAL.

Rats were chosen for the investigation of the action of deficient proteins, restricted mineral salts and accessory food factors on the production of amboceptor and agglutinins. These animals are very suitable for the purpose as they readily consume the experimental diet. For the study of the influence of quantitatively restricted diet on the complement content of the blood and the effect of a scorbutically restricted diet on the production of amboceptor and agglutinins guinea-pigs were employed, because these animals are well adapted for complement production and are very susceptible to scurvy. In both these instances their ordinary basal diet of oats and bran was so adjusted that the requisite deficiencies were effected without making their food unpalatable. The rats employed were mostly of the albino variety and were specially selected either from stock bred in this laboratory or from healthy stock bought outside. The animals were kept separately in wooden boxes with perforated zinc tops. The bottoms of the boxes, except in some experiments to be mentioned later, were covered with a thin layer of sawdust. The diets were delivered daily in the form of weighed balls of suitable consistency. These were weighed back the following morning to ascertain the amount eaten. The drinking water which was kept in glass dishes was changed twice a day. All the animals were closely watched during the experiment, especially for scabies, and were weighed on the average about twice a week.

The guinea-pigs too were chosen from healthy stock, great care being taken to ascertain that they had not previously been used for any inoculation experiments. These animals were kept separately in wire cages and received daily measured quantities of their respective foods in clean glass dishes. The food remnants were measured back the following day.

The rats' rations were made up by thoroughly mixing all the constituents of the diet by means of a mechanical mortar. The mixtures were brought up to a convenient consistency by the addition of water and olive oil. The diets were made up twice weekly and stored in a refrigerating room until they were wanted so that no ration was older than 3 or 4 days.

The basal ingredients of the rats' food consisted of caseinogen, except in a few cases in which that protein was not compatible with the object of the experiment, starch and a mixture of mineral salts.

The caseinogen was obtained from a commercial firm. It was pale yellow and of light texture. For experiments on the accessory food factors it was previously purified. The purification consisted of shaking the caseinogen twice with ether and once with 95 % alcohol for several hours and filtering and drying. By testing the purified caseinogen on rats it was found to be free from the antineuritic and the fat-soluble *A* accessory factors.

Pure wheaten starch was employed. It was not found necessary to purify this ingredient as it was free from both the antineuritic and the fat-soluble *A* accessory factors.

The salt mixture was made up of pure reagents in the same proportion as that used by McCollum and his colleagues, and was constituted as follows:

NaCl	1.73 g.	CaH ₄ (PO ₄) ₂ ·2H ₂ O	5.4
MgSO ₄ (anhydr.)	2.66	Calcium lactate	13.0
NaH ₂ (PO ₄)·H ₂ O	3.47	Ferric citrate	1.18
K ₂ HPO ₄	9.54				

The fat-soluble *A* factor was supplied in the form of purified butter fat. Pure butter was melted at 40° and centrifuged. The supernatant layer of fat was then carefully syphoned off leaving behind the caseinogen, water and impurities.

Autolysed yeast was added to the diet to supply the antineuritic. Ordinary top fermentation brewer's yeast was pressed out in order to remove the adhering wort. It was then introduced into flasks plugged with cotton wool and placed in the hot room at 37° until it had liquefied. The autolysed mixture was then filtered through a Buchner funnel by means of the suction pump. The resulting filtrate was brown and fairly transparent, and was rich in the antineuritic factor. As the autolysed yeast solution contains a good deal of the inorganic constituents of the yeast it was necessary to treat it further when used for the experiments in which the inorganic constituents of the diet were limited. This treatment will be described later.

The antiscorbutic factor was introduced into the diet in the form of lemon juice treated according to the method described by Harden and Zilva [1918]. This solution has the advantage of containing almost all the antiscorbutic content of the lemon juice associated with little extraneous matter and can be used in very concentrated form if necessary.

In studying the production of the antibodies the animals were fed on the restricted diet for several weeks, after which time they were immunised by

three injections of increased doses of organisms with an interval of 7 days between each injection. The animals were bled out a week after the last injection. The blood was then allowed to clot and centrifuged and the supernatant serum employed for the estimation of the agglutinin and amboceptor.

The immunisation was carried out with the typhoid organism "Simpson." Young 24-hour cultures were emulsified for the purpose and the emulsion brought up to a dilution of 1000 million per cc. It was then killed by heating it at 60° for $\frac{1}{2}$ hour. The same batch of emulsion which was kept in a refrigerating room was used for each group of experiments. The animals were weighed on the day of the injection and their doses were calculated in proportion to their weight. All the doses were brought up in volume with saline to 1 cc., and were injected subcutaneously in the abdominal region.

In estimating the agglutinins a "Simpson" typhoid emulsion containing 2000 million organisms per cc. was used. The different dilutions of the sera and the emulsion were kept at a temperature of 37° for 1 hour and read off 15 minutes after and again the following day.

The amboceptor was estimated by means of the complement fixation. For that purpose $\frac{1}{2}$ cc. of each dilution of the serum plus $\frac{1}{2}$ cc. of the "Simpson" emulsion of the same strength as that used for the agglutination plus $\frac{1}{2}$ cc. of complement were kept for one hour at 37°, after which time to each tube was added $\frac{1}{2}$ cc. of washed sheep's blood corpuscles and $\frac{1}{2}$ cc. of haemolytic amboceptor. The tubes were then kept for another hour at 37° and removed to the refrigerating room until the following morning, when the readings were taken. With each set two control tubes containing 1 in 25 serum without emulsion and a double quantity of emulsion, namely 1 cc., without serum were also set up. On each occasion a stock antityphoid serum was titred out for agglutinins and complement fixing bodies to demonstrate the activity of the reagents employed.

For the complement fresh guinea-pig serum diluted $\frac{1}{10}$ with saline was used. The haemolytic amboceptor was titrated the day of the estimation and double the registered strength necessary for haemolysis was employed.

Before proceeding with the experiments it was necessary to show that by injecting normal guinea-pigs and rats with the "Simpson" typhoid emulsion the production of agglutinins and amboceptor could be effected. It was also necessary to ascertain the number of injections required for the production of suitable quantities of agglutinins and amboceptor normally so that a restricted production of these antibodies could be demonstrated by comparison. For that purpose the following experiment was carried out.

Rat No. 55, weighing 54 g., received an injection of 1 cc. containing 40 million "Simpson" typhoid organisms, and was bled out a week after the injection.

Rat No. 56, weighing 46 g., received an injection of 1 cc. containing 40 million organisms and a week after another cc. containing 80 million organisms and was bled out a week after the second injection.

Guinea-pig No. 313, weighing 402 g., received an injection of 1 cc. containing 250 million organisms, and was bled out a week after the injection.

Guinea-pig No. 314, weighing 383 g., received an injection of 1 cc. containing 250 million organisms and another injection of 1 cc. a week after containing 500 million organisms. A week after the second injection this animal was bled out. Table I gives the agglutination and amboceptor titres of the above animals.

Table I.

In this and all the other tables + indicates agglutination or lysis.

<i>Agglutination.</i>								
	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200
Rat 55 (one injection)	+++	+++	+++	++	+	-	-	-
Rat 56 (two injections)	+++	+++	+++	+++	+++	++	++	-
Guinea-pig No. 313 (one injection)	+++	+++	+++	++	+	+	-	-
Guinea-pig No. 314 (two injections)	+++	+++	+++	+++	+++	++	+	-
Stock antityphoid serum	+++	+++	+++	+++	+++	+++	+++	++
<i>Amboceptor.</i>								
	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	
Rat 55 (one injection)	+++	+++	+++	+++	+++	+++	+++	
Rat 56 (two injections)	-	+	+++	+++	+++	+++	+++	
Guinea-pig No. 313 (one injection)	+++	+++	+++	+++	+++	+++	+++	
Guinea-pig No. 314 (two injections)	-		+	+++	+++	+++	+++	
Stock antityphoid	-	-	-	-	-	+	++	

It is seen from the above table that both in the normal guinea-pig and rat a very marked production of agglutinins was achieved with two injections. The amboceptor production did not respond so well but a decided change in the titre was effected with two injections. It was, therefore, decided to submit the experimental animals in this investigation to three injections with an interval of one week. The strength of the doses was to be calculated according to the body weight and was to be as follows: first injection 1 million organisms per g. body weight, second injection 2 million organisms per g. body weight, and third injection 4 million organisms per g. body weight. Lower dilutions of the serums, namely $\frac{1}{5}$ and $\frac{1}{10}$, were also to be titrated for the amboceptors.

As all the deficiency diets investigated could not conveniently be tested together, the experiments were divided into various sets. Several animals were put on each diet and their blood was pooled in order to obviate the error of idiosyncrasy. To make conditions as nearly as possible equal the weights, sex and colour of the animals of each set were kept the same. Each

set had a group of rats on a control diet which was theoretically complete and the daily ration consisted of the following ingredients:

Basal mixture	10.6 g.
Purified butter	1.7 „
Autolysed yeast	5 cc.
Olive oil	0.25 „
Antiscorbutic equivalent to lemon juice	5 „

The basal mixture was made up of 75 % of starch, 20 % of caseinogen and 5 % of salts.

The above diet contains all the known accessory factors. For the sake of convenience, the fat-soluble *A*, the antineuritic and the antiscorbutic factors will be referred to in this communication as *A*, *B* and *C* respectively and the theoretically complete control diet as the *ABC* diet.

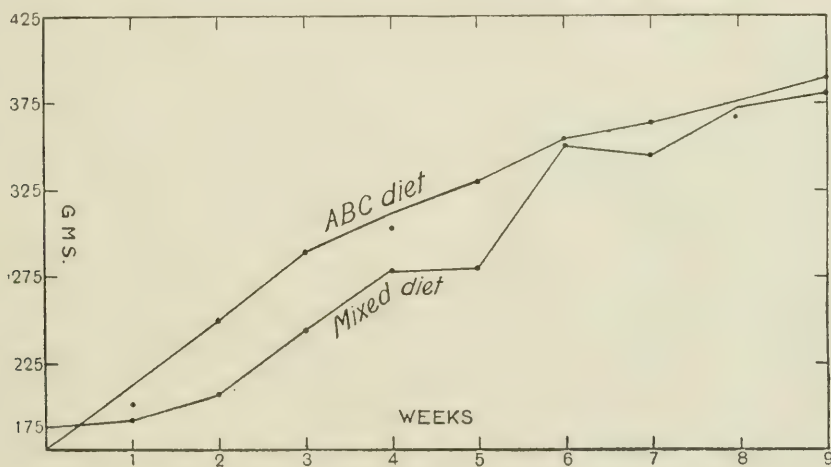


Fig. 1.

The first thing to ascertain was how rats fed on an *ABC* diet respond to injections in agglutinin and amboceptor formation in comparison with rats fed on an ordinary mixed diet. Two groups of rats each containing 3 animals were employed in this experiment. One group received an *ABC* diet, the other was fed on a mixed diet consisting of cabbage, boiled potatoes, scraps of bone and meat, biscuit meal, bread, carrots and boiled rice. After having subsisted for 6 weeks on the above diets the animals were immunised and bled out 9 weeks after the commencement of the experiment. The group receiving the *ABC* diet yielded 8 cc., the other group 9 cc. of blood. As will be seen from Fig. 1, which represents the growth curves of the two groups, the animals in both groups gained well in weight and the final weights were about the same in both cases. The increase in weight of the mixed diet group, however, was more erratic than that of the *ABC* group, which was most probably due to the lack of well-balanced uniformity in the diet of the former group, the

animals of which were left to choose their food from the offered mixture by instinct.

Table II gives the agglutination and amboceptor titres of the two groups. In both groups there was a very marked production of agglutinins and amboceptor, especially of the former, so that any differential production of these antibodies could not be missed. There is, however, no marked differentiation to be observed in the production of either of the antibodies. There is a slight difference in the amboceptor titres of the two groups, but it is of an order that may be neglected.

Table II.

	<i>Agglutination.</i>			<i>Amboceptor.</i>							
	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
<i>ABC</i> purified diet	+++	+	-	-	-	+	++	+++	+++	+++	+++
Mixed diet	+++	+	-	-	+	++	+++	+++	+++	+++	+++
Stock anti-typhoid serum	+++	+++	+++	-	-	-	-	-	-	+	++

Having ascertained that rats fed on an *ABC* diet respond to injections in the formation of agglutinins and amboceptor under the conditions described equally well as rats fed on a good mixed diet, it was possible to employ this diet as a control in the study of the various deficient diets.

It was previously mentioned that it has been definitely established that the inorganic constituents of the food are indispensable for existence. Lately Osborne and Mendel [1918] made a detailed study of the influence of various inorganic elements in nutrition and found that calcium and phosphorus were the only elements which when singly reduced to very low limits had a retarding effect on the growth of rats. In the present investigation the study of the influence of a deficiency of iron, calcium, potassium, phosphorus, chlorine and sodium on the production of the immune bodies was attempted. For this purpose in devising the diets precautions had to be taken in order to reduce the presence of the respective elements to a minimum. This was accomplished in all the cases with the exception of the sodium and chlorine, in which owing to an oversight the amounts of those elements consumed were rather higher than was intended. In all the groups wheaten starch was used as it was found to be fairly free from the inorganic constituents under investigation. Caseinogen was employed for the protein portion of the diet with the exception of the group which received a phosphorus-free diet, in which case egg-white was used instead. The egg-white was obtained from a commercial firm and did not contain any phosphorus.

The autolysed yeast could not be used in the ordinary way to supply the antineuritic factor as it contained a good proportion of the inorganic constituents of the yeast. It was, therefore, treated in a manner which reduced its salt content to a minimum. The procedure was as follows. Five parts by volume of autolysed yeast were mixed with 95 parts of absolute alcohol and

well stirred. A viscous precipitate containing mostly proteins and their degradation products and salts was formed. This was filtered off yielding a clear yellow-coloured filtrate. The alcoholic filtrate was then evaporated to dryness *in vacuo* at 40° and the residue obtained dissolved in a volume of distilled water equivalent to the original juice. This solution was tested on a polyneuritic pigeon and was found to be active. 3 cc. of this active solution was given in the daily ration and was sufficient to keep a control group of rats in good health. The treated lemon juice which was used to supply the anti-scorbutic factor in the diet contained so little inorganic matter that it could be neglected. The purified butter with the exception of the sodium chloride adhering to it contained little inorganic matter. Unfortunately the sodium chloride supplied by this source, although very low, was overlooked and, as has already been mentioned, made the diets intended to be deficient in these elements contain rather significant traces of sodium and chlorine. The inorganic constituents of the various diets were made up as follows:

Table III.

	Diet low in Iron	Diet low in Calcium	Diet low in Potassium
	g.	g.	g.
NaCl	4.6	4.6	4.6
MgSO ₄ (anhydrous)	7.4	7.4	7.4
NaH ₂ PO ₄	9.4	9.4	9.4
K ₂ HPO ₄	25.8	25.8	—
CaH ₂ (PO ₄) ₂	14.6	—	14.6
Ca lactate	35.3	—	35.3
Ferric citrate	—	3.2	3.2

All the above were made up with starch to 100 g.

Diet low in Sodium.

KCl	6.0	CaH ₂ (PO ₄) ₂	14.6
MgSO ₄ (anhydrous)	7.4	Ca lactate	35.3
K ₂ HPO ₄	25.8	Ferric citrate	3.2

Made up to 100 g. with starch.

Diet low in Chlorine.

MgSO ₄ (anhydrous)	7.4	CaH ₂ (PO ₄) ₂	14.6
NaH ₂ PO ₄	9.4	Ca lactate	35.3
K ₂ HPO ₄	25.8	Ferric citrate	3.2

Made up to 100 g. with starch.

Diet low in Phosphorus.

NaCl	4.6	CaCO ₃	6.3
MgSO ₄ (anhydrous)	7.4	Na ₂ CO ₃	4.2
K ₂ CO ₃	20.0	Ferric citrate	3.2
Ca lactate	35.3		

Made up to 100 g. with starch.

The basal mixtures for these experiments consisted of 80 % of starch, 20 % of protein and 5 % of the respective salt mixtures. The diets were made up in the same proportions as the *ABC* diet with the exception that only 3 cc. of the treated autolysed yeast was given.

Samples of the diets were analysed for the various elements with the following results:

Diet low in iron			Absent
..	..	calcium ...	0.014 %
..	..	potassium ...	Undeterminable traces
..	..	sodium ...	0.014 % calculated from Cl
..	..	chlorine ...	0.021 %
..	..	phosphorus	0.05 %

All the rats in this experiment received distilled water to drink and were housed in boxes containing perforated zinc floors. It was not advisable to use sawdust in this instance as some of it might have been consumed with the food and thus formed a source for inorganic constituents.

For convenience these experiments were divided into two sets. In the first set the deficiency of iron, calcium and potassium was investigated. In the second set the deficiency of sodium, chlorine and phosphorus was studied. Each set had a control group of animals. Each group of the first set consisted of five rats. These animals were kept 85 days on the experimental diets. The group on diet low in Ca yielded 16 cc., in K 20.5 cc., in Fe 21.5 cc. and the control 19 cc. of blood. Fig. 2 shows that the animals receiving the diets deficient in Fe and K grew almost as well as the control group of rats, although the K group showed a slight decline towards the end of the experiment. On the other hand, the group deficient in Ca showed a decided restriction in their development. By examining Table IV it will be seen, however, that the agglutinins and amboceptor titres are almost identical in all the groups.

The second set of animals had 3 rats in each group. It will be seen from Fig. 3, that although the groups deficient in sodium and chlorine developed as well as the control in this set, the group receiving the diet low in phosphorus not only failed to show any growth but actually declined. In order to obviate the loss of the animals in this group the experiment was terminated after 54 days. The following quantities of blood were obtained. The sodium group yielded 9 cc., the chlorine 10 cc., the phosphorus 9 cc. and the control 9 cc. of blood. It is to be noticed that the phosphorus group although the lightest in weight yielded as much blood as the other groups. By examining Table V it is seen that no differentiation in the agglutinins and amboceptor formation in the groups which received the diets deficient in Na and Cl could be observed. The animals which were fed on the diet low in phosphorus showed a decidedly lower titre in the agglutinins and amboceptor. In fact hardly any amboceptor formation took place in this group. As will be seen from the other experiments which follow, this was the only group that showed a lower amboceptor titre.

In arranging the above diets it was impossible to eliminate from the basal

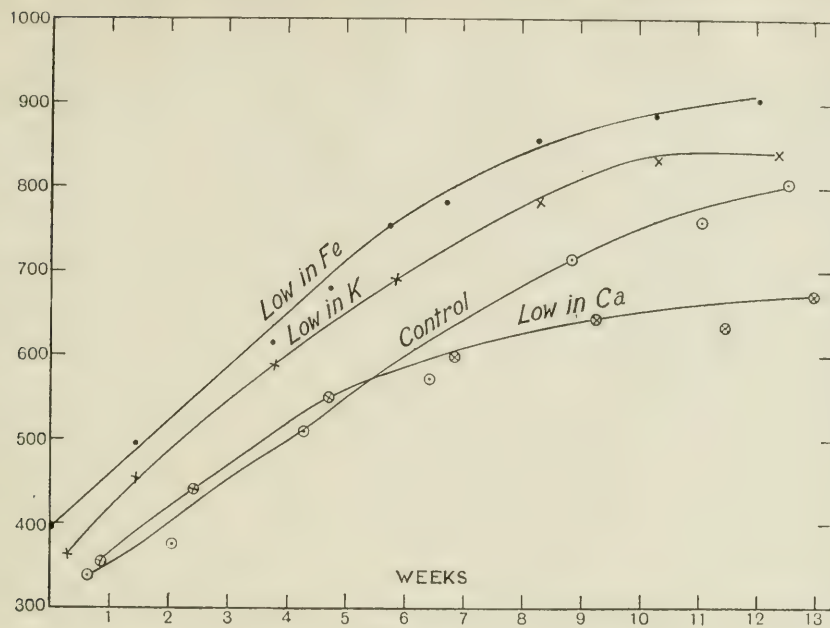


Fig. 2

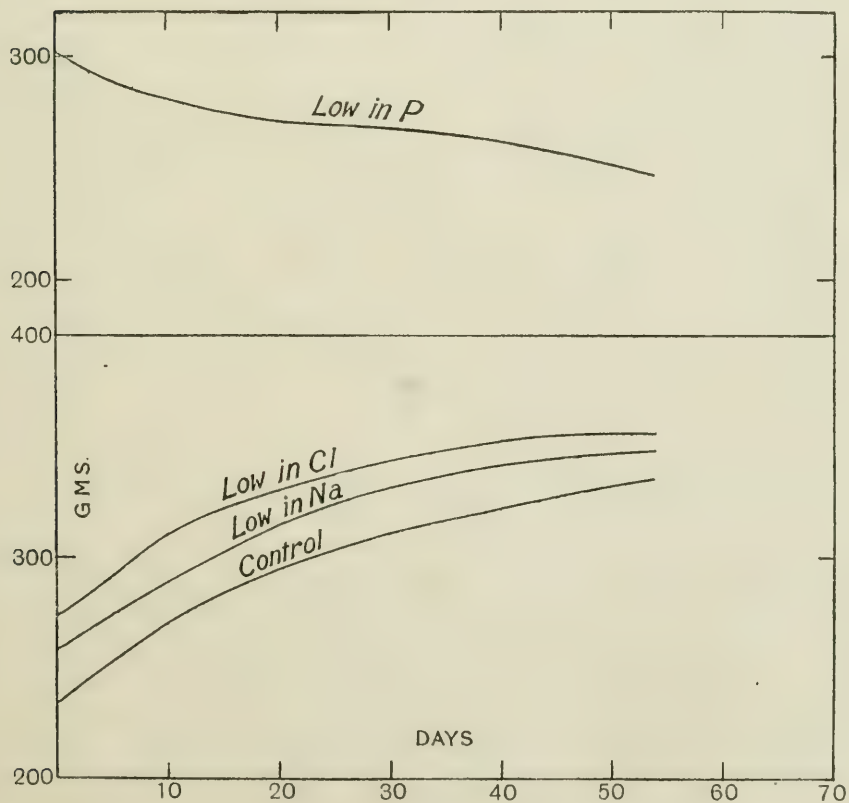


Fig. 3

salt mixture any one element without at the same time altering quantitatively the content of some of the other constituents. This alteration was not of an important character as care was taken not to establish a second deficiency, and in view of the results obtained it can be ignored.

Table IV.

	<i>Agglutination.</i>					<i>Amboceptor.</i>				
	1/800	1/1600	1/3200	1/6400	1/25	1/50	1/100	1/200	1/400	1/800
Control diet	+++	+++	+++	+	-	++	+++	+++	+++	+++
Ca-free diet	+++	+++	+++	+++	-	+	+++	+++	+++	+++
K-free diet	+++	++	-	-	-	++	+++	+++	+++	+++
Fe-free diet	+++	+++	++	-	-	+++	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	+++	-	-	-	-	++	+++

Table V.

			<i>Agglutination.</i>						
			1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800
Control diet	+++	+++	+++	++	++	++	+
Diet low in phosphorus			+++	++	+	trace	-	-	-
Diet low in sodium	...		+++	+++	+++	+++	++	+	-
Diet low in chlorine	...		+++	+++	+++	+++	++	+	-
Stock antityphoid serum	...		+++	+++	+++	+++	++	++	+

<i>Amboceptor.</i>										
		1/5	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
Control diet		+	++	++	+++	trace +++	+++	+++	+++	+++
Diet low in phosphorus	trace	trace								
	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Diet low in sodium						trace				
	-	-	+	+	+++	+++	+++	+++	+++	+++
Diet low in chlorine					trace					
	-	-	+	+++	+++	+++	+++	+++	+++	+++
Stock antityphoid serum										
	-	-	-	-	-	+	++	++	++	+++

On examining the composition of the diet low in phosphorus it is seen that two other factors were also altered. The salt mixture was slightly more alkaline owing to the higher content of the carbonates of sodium and potassium which replaced the phosphates of those salts, and the caseinogen was replaced by egg-white, which from observations made in control experiments does not seem to be as good a source of protein as caseinogen. As the result obtained with this group is interesting the subject will have to be studied further before definite conclusions can be arrived at.

The next thing to be investigated was the influence of diets deficient in certain amino-acids on the production of the antibodies in question. Two sets

of experiments were devoted to this subject. In the first set a low intake of caseinogen was studied, in the second the action of gliadin as the sole source of protein in the diet.

Osborne and Mendel [1915] were the first to demonstrate that if caseinogen formed 18 % of a diet it was adequate to ensure normal growth in rats; if, however, the content of that protein in the diet was reduced to 9 % the rate of growth of the animals was greatly impaired. The explanation was advanced that cystine was the limiting factor, caseinogen having a low cystine content. When it forms as much as 18 % of the diet the amount of cystine supplied through its medium is sufficient to satisfy the requirements of the animals, when, however, it forms only 9 % of the diet the amount of cystine consumed becomes deficient and manifests itself by the restricted growth of the animals. This explanation was confirmed by the fact that the addition of cystine to a low caseinogen diet raised its nutritive value to the same level as that of lactalbumin.

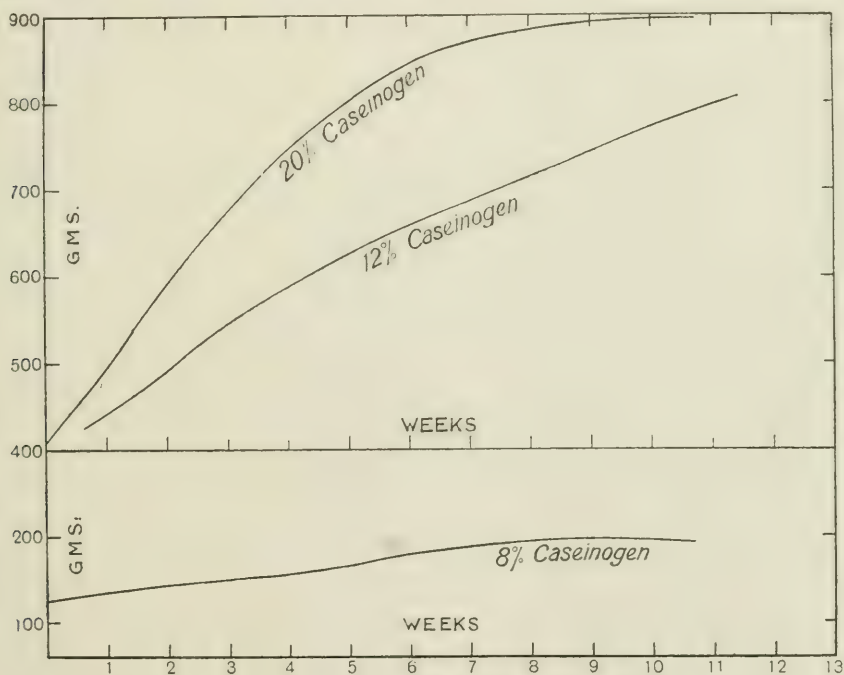


Fig. 4

In order to study the deficiency of cystine, diets of low caseinogen content were used in this investigation. Three groups of rats receiving 20 %, 12 % and 8 % respectively, were used. With the exception of the varied quantities of the caseinogen and starch the diets were in other respects similar to the *ABC* diets. Six animals were started in each group, but only two survived in the group receiving a diet with 8 % of caseinogen. Fig. 4 gives the weight curves of the three groups. It is seen that the group which received the 8 %

caseinogen diet did not grow at all. The group fed on the 12 % caseinogen diet grew but did not do as well as those on the 20 % diet. When killed 23 cc. of blood was collected from the group of six rats fed on the 20 % caseinogen diet, 15 cc. from the group of six rats which received 12 % diet, and 4½ cc. from the group of two rats which survived on the 8 % diet. Table VI shows that no marked difference can be observed either in the agglutinins or in the amboceptor formation of the three groups in spite of the restricted growth which the groups receiving 12 % and 8 % of caseinogen manifested.

Table VI.

	<i>Agglutination.</i>					<i>Amboceptor.</i>		
	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200
Diet containing 20 % caseinogen	+++	+++	++	—	±	+++	+++	+++
Diet containing 12 % caseinogen	+++	+++	—	—	±	+++	+++	+++
Diet containing 8 % caseinogen	+++	++	—	—	++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	—	—	+	++	+++

According to Osborne and Mendel [1914, 1916], gliadin as the sole protein in a diet suffices to maintain rats, but is insufficient to promote growth in young animals unless it is supplemented by lysine, an amino-acid which this protein does not contain. The influence of gliadin was studied in this investigation on a set of rats containing one group receiving an *ABC* diet with caseinogen as the protein and another group having the caseinogen replaced by gliadin.

The gliadin was prepared from wheat flour by the method described by Osborne [1910]. The product was precipitated twice with water and once with absolute alcohol and was thus carefully purified. The basal mixtures in these experiments were made up as follows:

Control diet.

Starch 77 %, Caseinogen 18 %, Salts 5 %.

Gliadin diet.

Starch 77 %, Gliadin 18 %, Salts 5 %.

The above basal mixtures were mixed with the necessary ingredients in proportion already described to form an *ABC* diet.

The weight curves of the two groups (Fig. 5) show that rats which were fed on the gliadin diet failed to grow, thus confirming the observations of Osborne and Mendel. Six animals were started in each group, but only four survived in the gliadin group and consequently the blood of four animals in each of the above groups was pooled for examination. These rats were immunised after 38 days instead of 6 weeks as it was feared that more animals

in the gliadin group might die if the experiment were prolonged. The animals in the gliadin group yielded 9 cc. of blood, the four animals in the control group yielded 16 cc. of blood. Table VII shows that the gliadin diet made no difference to the agglutinin and amboceptor production, although the animals in this group showed no growth owing to the restriction in the diet. The view that the nutritive restriction of gliadin is due to the lack of lysine is not altogether supported by the observations of McCollum, Simmonds and Pitz [1917] and Geiling [1917]. The former found that when maize kernel, which is poor in lysine, is supplemented by gelatin, which is very rich in that amino-acid, the nutritive value of the maize is not enhanced. The latter is led to believe by his investigation that lysine is not indispensable for existence of the mouse. Whatever the deficiency of gliadin may be due to it does not inhibit the production of agglutinins and amboceptor.

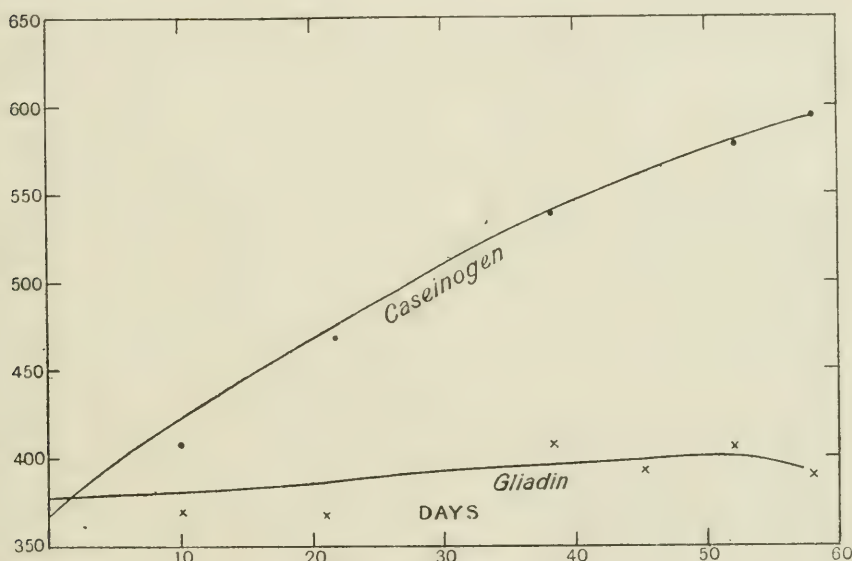


Fig. 5

Table VII.

	Agglutination.				Amboceptor.				
	1/800	1/1600	1/3200	1/6400	1/100	1/200	1/400	1/800	1/1600
Control diet containing 18 % caseinogen	+++	+	-	-	-	+	+++	+++	+++
Diet containing 18 % gliadin	+++	++	-	-	-	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	++	-	-	-	+	++	+++

The following sets of experiments were devoted to the investigation of the influence of the lack of the accessory factors. Observations were carried out on animals subsisting on diets deficient in the antineuritic, fat-soluble *A*, and antiscorbutic factors. McCollum and Davies have revealed by their researches that rats lacking the antineuritic factor in their diet fail to grow and die after about 6 weeks, often manifesting decided neuritic lesions. This was confirmed by Drummond [1917] and by Harden and Zilva [1918, 2]. If these animals are deprived of the fat-soluble *A* factor they may show a certain amount of growth during the initial stage of the experiment, but eventually also cease to develop and die. In most of these cases the animals display a condition of the eyes resembling xerophthalmia. Although rats do not actually manifest scorbutic lesions when kept on a scorbutic diet there seems according to Harden and Zilva [1918, 2] and Drummond [1919] to be evidence that they do not thrive as well on such diets as they do when the antiscorbutic factor is supplied. The standard *ABC* diet used in these experiments contained all the three factors. The *AB* diet was deprived of the treated lemon juice, the *BC* diet of the butter fat, and the *AC* diet of the autolysed yeast. They were made up as follows:

AB diet daily ration.

Basal mixture	10 g.	Autolysed yeast	5 c.c.
Purified butter	1.85 g.	Olive oil	2.5 c.c.

BC diet daily ration.

Basal mixture	12 g.	Antiscorbutic, equivalent to			
Autolysed yeast	5 c.c.	lemon juice...	5 c.c.
				Olive oil	0.75 c.c.

AC diet daily ration.

Basal mixture	10 g.	Centrifuged butter fat	...	1.8 g.
Antiscorbutic, equivalent to				Olive oil	...	1.6 g.
lemon juice	5 c.c.			

The basal mixtures in all the above diets were of the same composition as the control *ABC* diet already described.

As these experiments were intended to last 9 weeks the majority of the animals subsisting on the *BC* and *AC* diets would by that time have succumbed to the deficiency and thus frustrated the object of the experiment. It was, therefore, found necessary to add at times small quantities of butter and autolysed yeast to the respective groups in order to prolong the existence of the animals, but the quantities of these addenda had to be of an order insufficient to promote normal growth. It will be seen from Fig. 6 that the *ABC* group grew best, the *AB* group did almost as well, while the *BC* group and more so the *AC* group show restricted development. Both these groups exhibited the characteristic appearance of animals existing on restricted diets. Five rats were used in each group of this experiment. By examining Table VIII it is seen that there was no change in the amboceptor production, but the

agglutinin titres showed a great variation, none of the groups receiving the deficient diets producing as much as the control group. Taking this as an indication that the absence of the accessory factors from the diets influences the agglutinin production in the animals it became desirable to repeat the experiment. A set of animals, therefore, similar to the above were again submitted to the same treatment as the first, but as will be seen from Table IX no differentiation in the agglutinin titres was obtained the second time. This would suggest that the impaired agglutinin formation could not be ascribed to the consumption of the deficient diets. Another experiment about to be described gives further support to this view.

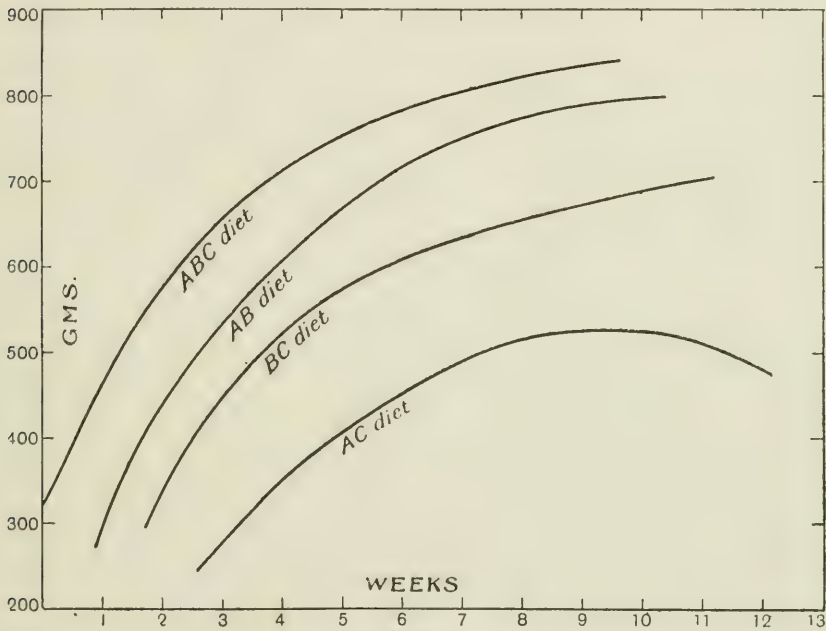


Fig. 6

Table VIII.

Agglutination.

Amboceptor.

	1/100	1/200	1/400	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
ABC purified diet	+++	+++	+++	+++	+	-	-	++	+++	+++	+++	+++	+++	+++
AB purified diet	+++	+++	+	-	-	-	-	-	++	+++	+++	+++	+++	+++
BC purified diet	+++	+++	+	-	-	-	-	-	+	++	+++	+++	+++	+++
AC purified diet	+++	+	-	-	-	-	-	+++	+++	+++	+++	+++	+++	+++
Stock antity-phoid serum	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-

Table IX.

	<i>Agglutination.</i>			<i>Amboceptor.</i>						
	1/3200	1/6400	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
<i>ABC</i> purified diet	+++	—	—	—	—	—	+++	+++	+++	+++
<i>AB</i> purified diet	+++	+	—	—	—	+++	+++	+++	—	+++
<i>BC</i> purified diet	+++	—	—	+	+++	+++	+++	+++	+++	+++
<i>AC</i> purified diet	—	—	—	—	—	—	+++	+++	+++	+++
Stock anti-typhoid serum	+++	+++	—	—	—	—	+	+	+++	+++

It has already been mentioned that rats kept on a scorbutic diet do not show scorbutic lesions. It was, therefore, of interest to study the influence of the antiscorbutic deficiency on the production of the antibodies in guinea-pigs, which are very susceptible to scurvy. These animals, if kept on a scorbutic diet, manifest signs of scurvy after about 9 days and succumb to the disease about a month after. After about 15 days these animals usually cease to grow, and eventually decline in weight. It is possible by administration of small doses of antiscorbutics just to maintain the weights of the guinea-pigs and keep the animals alive in this way for months. Animals in such a condition are decidedly scorbutic, as can be proved by chloroforming them and holding a post mortem examination. By taking advantage of this fact it was possible to investigate the subject on guinea-pigs. Three groups of guinea-pigs were employed in this experiment. One group received a basal diet of oats and bran and 40 cc. of autoclaved milk and occasional doses of orange juice given *per os* at intervals sufficient to maintain the weight of the animals constant. The second group received a quantitatively restricted mixed diet containing plenty of antiscorbutics. The food was given in quantities sufficient to maintain the weights of the animals, but insufficient to permit growth. It consisted of a daily ration of 5 g. of cabbage, 10-20 g. of bran and 30 cc. of fresh milk containing 10 cc. of treated lemon juice. The third group received oats, bran, milk, cabbage and roots *ad lib*. All the animals were kept 73 days on their diets. The animals received 250 million organisms in the first, 500 million in the second, and 1000 million in the third injection per 400 g. of body weight. Fig. 7 shows the weight curves of the three groups. Four animals were bled out in each group and the unrestricted mixed diet group yielded 48 cc., the restricted mixed diet group 31 cc. and the scorbutic group 26 cc. All the animals of the scorbutic group showed well-marked signs of scurvy, whereas all the animals of the other groups were entirely free from any trace of scorbutic lesions. The agglutination and amboceptor titres, which are represented in Table X, do not show any differentiation in the three groups. These results go further to support the view that the impaired agglutinin production observed

in the first set of rats fed on the diets deficient in the accessory factors was not due to the diet.

It is well known that guinea-pigs form a good source of complement and it was of interest to ascertain whether, if these animals are restricted in their diet, the complement activity of their blood is impaired. The blood of the animals of the three groups described above was therefore tested for its complement content. Varying quantities of the serums were added to tubes containing a haemolytic amboceptor and washed blood corpuscles. As Table XI discloses, no variations in the titre could be observed, thus showing that the bloods of the three groups were of almost the same activity.

Table X.

	<i>Agglutination</i>					<i>Amboceptor</i>							
	1/200	1/400	1/800	1/1600	1/3200	1/10	1/25	1/50	1/100	1/200	1/400	1/800	1/1600
Scorbutic diet	+++	+	-	-	-	-	+	++	+++	+++	+++	+++	+++
Restricted mixed diet	+++	++	-	-	-	-	+	++	+++	+++	+++	+++	+++
Unrestricted mixed diet	+++	++	-	-	-	-	-	+	+++	+++	+++	+++	+++
Stock antityphoid serum	+++	+++	+++	+++	+++	-	-	-	-	-	-	-	-

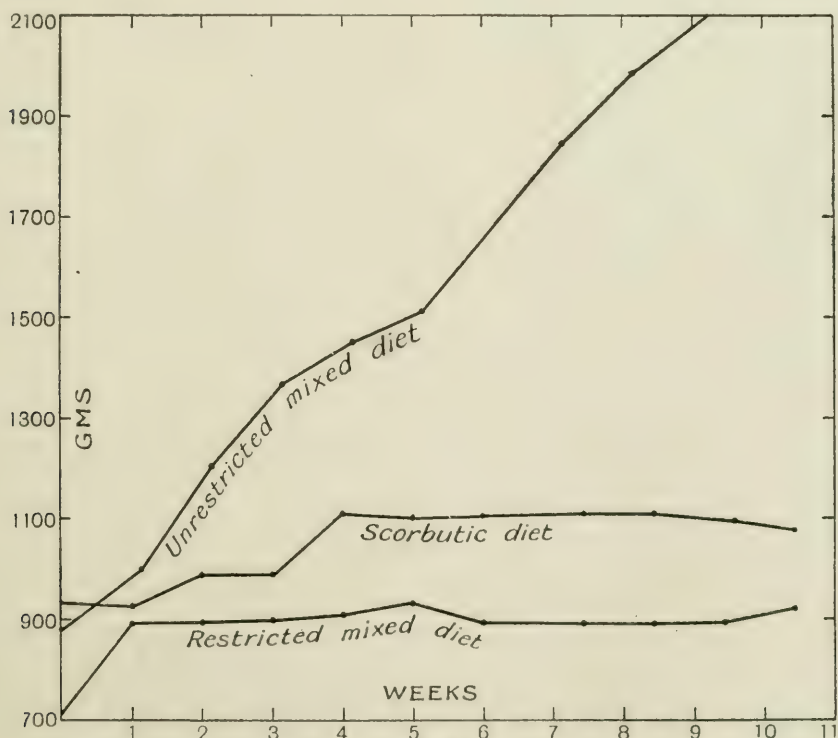


Fig. 7

Table XI.

Complement $\frac{1}{4}$	0.2 c.c.	0.1 c.c.	0.05 c.c.
Scorbutic diet ...	+++	++	
Restricted mixed diet ...	+++	++	
Unrestricted mixed diet ...	+++		

Table XII.

Complement $\frac{1}{4}$	0.2 c.c.	0.1 c.c.	0.05 c.c.	0.025 c.c.	0.01 c.c.
Restricted mixed diet ...	+++	+++	++	-	-
Unrestricted mixed diet ...	+++	+++	-	-	-

Another set of experiments on the subject was instituted with the object of ascertaining whether a longer period of dietetic restriction would influence the activity of the complement.



Fig. 8

Two groups of six guinea-pigs each, kept on the experimental diet for 202 days, were investigated. One group received a quantitatively restricted mixed diet and was of the same nature as the one described above. The other group received a mixed diet *ad lib*. Fig. 8 shows their weight curves which are similar in character as those of the previous experiment. At the end of the experiment these animals were bled out and their blood was pooled. The restricted mixed diet group yielded 67 cc. and the unrestricted mixed diet group 111 cc. of blood. As will be seen from Table XII the prolonged dietetic restriction did not influence the complement content of the blood of these animals.

CONCLUSION.

Of all the groups of animals tried on the various deficient diets only the group which received a diet deficient in phosphorus showed a decidedly poorer response to the inoculations in the production of amboceptor and agglutinins. One set of animals subsisting on diets deficient in the accessory food factors showed a low production of agglutinins only, but as this did not agree with the results obtained on guinea-pigs fed on a scorbutic diet this particular experiment was repeated. The results obtained on repetition failed to confirm the differential production of the agglutinins in rats fed on diets deficient in the accessory food factors. On the other hand, the majority of the groups of animals fed on deficient diets failed to show any difference in the production of both agglutinins and amboceptor. This becomes more remarkable when it is seen that some of the groups owing to the dietetic deficiency manifested very restricted growth or no growth at all and were altogether in a much poorer condition than the control animals. It is true that none of these experiments has been repeated, but we have several instances in this investigation where owing to various nutritional deficiencies the animals have displayed a very inferior condition to the control rats fed on a theoretically complete diet and yet have shown no differential production of the antibodies. The results of the groups fed on the 12 % and 8 % caseinogen diets, on the diet low in calcium and on the gliadin diet may be cited in this connection. It is, therefore, interesting that the group of animals fed on a diet low in phosphorus should be the only one showing a well marked differentiation in their agglutinins and amboceptor titres. This, of course, invites further investigation in order to establish the fact definitely. The results of the experiment can at present only be considered as an indication.

This investigation has only dealt with two antibodies produced under certain definite conditions. What actual bearing these antibodies have on immunity is not well established, but evidently they have some unexplained indirect connection with it, as the Widal's reaction seems to suggest in the case of agglutination. On the whole the subject is still very obscure. In the case of bacteriolysins it is known that as a result of infection or inoculation the corresponding bacteriolytic amboceptors are formed. This is demonstrated by bacteriolysis *in vitro* and *in vivo*; yet this bacteriolytic power of the blood of the organism does not remain, while the increased resistance to the disease is sometimes established for years. It is, therefore, plain that the actual capacity of the animal for antibody formation cannot be taken as a definite indication of the power to resist disease, although in conjunction with other observations it may give valuable information on the subject. A systematic study of the influence of nutrition on the production of immunity, although complex and laborious, is urgently called for and may not only prove to be of direct clinical value but may elucidate many obscure points in the complicated mechanism of immunity.

SUMMARY.

The influence of deficient diets on the production of agglutinins and amboceptor in rats was investigated.

The following deficiencies were studied.

1. Diets low in iron, calcium, potassium, sodium, chlorine and phosphorus.
2. Diets containing 12 % and 8 % of caseinogen as a source of protein.
3. Diets containing 18 % of gliadin as the sole source of protein.
4. Diets deficient in each of the three accessory food factors.

Although several of the deficiencies became manifest by the restricted growth and the poor condition of the animals, no differentiation in the titres of the agglutinins and amboceptor could be recorded, except in the group receiving the diet low in phosphorus. Guinea-pigs fed on an unrestricted mixed diet, quantitatively restricted mixed diet and a scorbutic diet showed no differentiation in the amboceptor and agglutinin titres, and in the complement activity of the blood.

Guinea-pigs kept on an unrestricted mixed diet and a quantitatively restricted diet for about 6 months showed no differentiation in the complement activity of the blood.

In conclusion I wish to express my gratitude to Dr H. Schütze for the valuable help and advice he has given me in connection with the bacteriological work of this investigation. Dr Schütze has been good enough to check independently all the readings of the agglutinin and amboceptor titres.

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XXI. THE NOMENCLATURE OF BLOOD PIGMENT AND ITS DERIVATIVES.

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(Received May 8th, 1919.)

ALTHOUGH a clear knowledge of blood pigment and its derivatives is essential to the student, teaching experience has shown how difficult it is to impress so many details upon his mind, and this is mainly because the present nomenclature requires a mnemonic effort on his part. It is perplexing because it does not logically express the relationship between the natural pigment and its derivatives. The reason for this state of things is partly the gradual acquisition of knowledge of these relationships, and partly the arbitrary way in which names were chosen by Hoppe-Seyler, whose authority at the time was sufficiently great to force his nomenclature into the scientific literature of his own and other nations.

Hoppe-Seyler cannot be acquitted of the charge of having ignored, as regards nomenclature, the claims of previous observers. Of this we may take the following examples.

(i) Stokes [1864] in the same paper in which he described the discovery of the spectrum of reduced blood-pigment, proposed on Professor Sharpey's suggestion the name "cruorin" and "reduced cruorin" (scarlet cruorin and purple cruorin). Four years later Hoppe-Seyler [1868] confirmed this discovery in the third part of his full paper on blood pigments. In some previous preliminary communications he had used Berzelius' old name "haematoglobulin¹," shortened sometimes into "haemoglobin." Under the influence of Stokes' discovery he was obliged to use a second term and employed the somewhat clumsy name "oxyhaemoglobin," promiscuously with haemoglobin, in the second edition of his text-book (1865). He was thus enabled in 1868 to ignore Stokes' nomenclature, especially since Stokes made no further communication on the subject.

(ii) To Thudichum [1867] belongs the credit of having first illustrated and described the spectrum of the pigment obtained by the action of con-

¹ From Liebreich's original manuscript notes of Hoppe-Seyler's lectures in 1863-64 (in the possession of one of us [O.R.]) it is evident that Hoppe, as he was then called, used at that time the term "haematoglobulin" exclusively.

centrated sulphuric acid on blood pigment which he called "eruentine." This fact is not even mentioned by Hoppe-Seyler [1871] when he re-labelled this substance "haematoporphyrin."

(iii) A further instance is the introduction of the term "haemochromogen" to which we shall return directly.

A nomenclature logically expressing the relationship of these substances would start from a short name for the iron-free pigment, and designate its precursors containing iron, protein, and oxygen by suitable prefixes. But an attempt to remodel the nomenclature drastically on such lines (however desirable it would be) would only lead to confusion, for the Hoppe-Seyler nomenclature is at present firmly established in physiological literature. A recent attempt to remodel anatomical nomenclature in a wholesale manner has for corresponding reasons proved to be a failure.

In one point, however, a change is possible, and strongly indicated. The name "haemochromogen," one of the greatest stumbling blocks of the student, should be deleted. The reasons for this are two: first, it is misleading, and secondly it is unnecessary.

It is misleading because the substance is not a "chromogen" in the modern sense, but a coloured substance. The term, therefore, is ill-chosen, and does not in the least indicate its nature.

It is unnecessary because it was adopted to replace another term introduced by its discoverer, and this original name (reduced haematin) has not only priority, but expresses its nature. It was Stokes [1864] who discovered that "brown" alkaline haematin is changed by reducing agents into "red" reduced haematin possessing a characteristic spectrum. Stokes not only discovered the spectrum of reduced haematin, but was also the first to suggest its use for forensic purposes, so characteristic is it, even when the substance is present in minute quantities. It was not until seven years later that Hoppe-Seyler [1871] confirmed Stokes' observation, but gave the substance in question the name of haemochromogen, although he admitted that haemochromogen in alkaline solution is identical with the reduced haematin of Stokes.

The reasons for the adoption of the new term urged by Hoppe-Seyler, are in Gamgee's words [1880]:

"Haemochromogen is a mere product of decomposition of haemoglobin, whilst haematin is an oxidised product of decomposition."

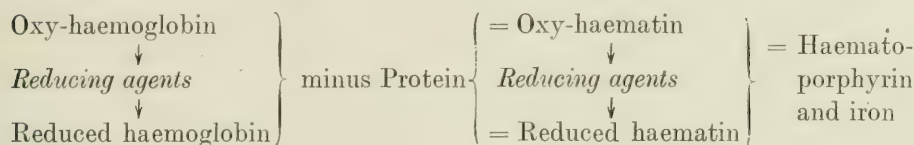
This appears to be a verbal quibble and its justification is entirely lost since even Hoppe-Seyler seems to have dropped his claim of having obtained the substance in crystalline condition directly from haemoglobin [see note by Thierfelder, 1903].

Nearly half a century has elapsed since the introduction of the word haemochromogen, and during this time no evidence has been brought forward to show that reduced haematin and haemochromogen are not one and the same substance. The time has arrived when it is no longer necessary to overburden the nomenclature with a special name. No special name has been found

necessary for reduced haemoglobin: one of us who writes text-books intends in the future to use Stokes' original term, as it is not misleading and expresses the logical relation of the substance to haematin, or, as it will now be necessary to call it, oxy-haematin.

The suggestion is not entirely a new one; Preyer [1871], Linossier [1887], and Abderhalden [1912] have made similar protests.

If this small amendment is made in the nomenclature, we feel sure of its benefit to students, for the relationship of the pigments can then be expressed by the following simple scheme:



As reducing agents the most frequently employed are either ammonium sulphide or ferrous ammonium tartrate, both originally introduced by Stokes [1864]. Both have disadvantages which it is unnecessary to recapitulate here, for they are well known to those who conduct classes of practical physiology. But perhaps the greatest disadvantage they possess is that, unless very carefully applied, the colour change from arterial to venous blood is not convincing to the junior student. We recommend a solution of sodium hydrosulphite ($\text{Na}_2\text{S}_2\text{O}_4$) as an excellent reducing agent for this purpose. One drop of a 10 per cent. solution is quite sufficient for a test-tube full of dilute oxy-haemoglobin, or oxy-haematin, such as is used in spectroscopic examinations of these substances. The solution itself is colourless, the application of heat is unnecessary, the colour change is immediate and the spectrum is not disturbed by secondary reactions. The employment of this reagent is not new and it seems to have been used about fifty years ago by Schützenberger, its discoverer. He, however, knew the substance only as an unstable solution which had to be prepared from sodium bisulphite by reduction with zinc. The substance is now manufactured on a large scale in this country for the dyeing industry and is obtainable as a dry powder which keeps well. It has the advantage of cheapness over equally satisfactory reducing agents such as hydrazine or hydroxylamine. A 10 per cent. solution of it remains active for many days when kept in a dropping bottle with vaselined stopper.

SUMMARY.

It is suggested that the misleading and unnecessary word haemochromogen be deleted from our vocabulary. Its original name, reduced haematin, should be used instead, and the substance hitherto termed haematin should now be called oxy-haematin.

This would bring the terms into line with reduced and oxy-haemoglobin respectively, and so make the student's work easier in remembering the relationship between blood pigment and its derivatives.

The use of sodium hydrosulphite as a reducing agent is recommended.

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XXII. THE ANTI-SCORBUTIC VALUE OF DRY AND GERMINATED SEEDS.

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(Received May 13th, 1919.)

SCURVY is always a menace to any community of people who are separated from adequate supplies of fresh food, whether in tropical or arctic climates. Such it proved to be to our army in Mesopotamia in 1915 and 1916 (*Mesopotamia Commission Report*, 1917), especially among the Indian troops. In consequence of this outbreak, a group of investigators in this Institute undertook an investigation of foodstuffs to determine their relative value as anti-scorbutics and especially to discover some effective material which could easily be transported and would not readily deteriorate. It has been known for centuries by practical experience of human diets, and has recently been proved experimentally [Holst and Frölich, 1912; Delf, 1918] that vegetables of high anti-scorbutic potency become useless for the prevention of scurvy if they are dried and thus rendered suitable for transport.

The most likely solution of the problem had already been indicated by Fürst [1912]. In the series of classical enquiries into experimental scurvy initiated by Holst in the University of Christiania, the work of Fürst showed that anti-scorbutic stuff was developed in substantial quantity during the first days of germination of various seeds. Peas, beans and other pulses, containing as they do only about 10 % of water, are eminently suited for transport, do not readily deteriorate and can be germinated for use where and when required. They form moreover a large part of the normal diet of the Indian soldier.

Experiments at this Institute with guinea-pigs on a diet consisting mainly of peas or lentils confirmed Fürst's results, and these preliminary trials were published in brief by Chick and Hume [1917] in whose paper directions were given for the germination of the dry seeds on active service. The subject appeared, however, to deserve more detailed examination, especially in respect of the anti-scorbutic value of germinated seeds in comparison with other foodstuffs and of its destruction by ordinary methods of cooking. The work undertaken to complete and amplify the earlier trials is set out in the following pages. Protocols of the individual animal experiments are given in the tables, which in many cases contain results of the earlier and later experiments side by side.

I. EXPERIMENTS OF FÜRST.

Guinea-pigs fed exclusively on cereals and water, the routine "scurvy diet" in Holst's laboratory, die of acute scurvy in 20 to 30 days. Fürst [1912] showed that when dry pulses were substituted for the cereals, scurvy also occurred, though in a less acute form, and life was to some extent prolonged. Thus five animals on a diet of dry yellow peas and water died of scurvy in 29 to 55 days; on dry green peas three animals out of four showed definite scurvy in 57 to 64 days, while the fourth lived 131 days without signs of the disease. No growth occurred in any of these animals, which indeed lost weight considerably. Fürst's experiments with germinated seeds were made with barley, oats, peas and lentils. These were soaked in water for 24 hours and afterwards kept moist with access of air and allowed to germinate at summer temperature for two or three days, when sprouts were distinctly visible. In this condition the seeds showed distinct anti-scorbutic properties. Thus two animals fed on water and sprouted barley lived 52 and 92 days without any sign of scurvy and four animals on sprouted oats lived 35 to 78 days and only one had symptoms. With peas and lentils even better results were obtained: four animals on germinated green peas lived 143 to 182 days and four on germinated lentils 89 to 183 days without signs of scurvy.

Growth of animals was also defective with germinated seeds, increase of weight taking place only with green peas.

Fürst also made the interesting observation that if germinated barley was dried at 37° the anti-scorbutic properties gained during germination were lost.

II. METHODS ADOPTED IN THE PRESENT RESEARCH.

The method used in this department in the study of experimental scurvy differs from that employed by Holst and his colleagues in two important points. In the first place the uneaten residues of food are collected and weighed each day, so that the actual intake of food is measured as well as the amount of the offered ration; this seems to us essential in any quantitative nutritional experiments. In the second place a daily ration of cow's milk, autoclaved for one hour at 120° C. (to reduce its original content of anti-scorbutic vitamine to a minimum) is in many cases added to the diet to improve its nutritive value without appreciably affecting the supply of antiscorbutic accessory factor.

In the earlier experiments, the basal diet of the guinea-pigs was water with oats and bran *ad libitum*, on which diet death from acute scurvy was found to take place in from 20 to 30 days. To this diet a weighed ration was added daily of the substance whose anti-scorbutic value was to be investigated. When peas or lentils, dry, soaked or germinated, were added to this basal diet, there was little or no growth, irrespective of whether scurvy was or was not prevented. The addition of autoclaved milk, however, helped to supply the missing growth-promoting substances. On a diet of oats, wheaten bran and autoclaved milk, normal growth and development took place if an adequate

amount of the anti-scorbutic factor were supplied in addition. When the supply of the antiscorbutic factor was inadequate, the initial growth was soon checked, scurvy symptoms were observed and death from scurvy occurred in a time only slightly longer than when the diet consisted of grain and water only. (See Table VI.)

The symptoms of guinea-pig scurvy have recently been described in detail [Chick, Hume and Skelton, 1918; Delf, 1918] and it is unnecessary to recapitulate them here.

Dry "Clipper" brand green peas (*Pisum sativum*) and ordinary brown lentils (*Lens esculenta*), unsplit and unhusked, were selected for the experiments. The latter, as "masoor dhal," is a common article of the native Indian diet. Most of the work was done with whole lentils purchased in the ordinary market; they were obtained with difficulty as it is usual to mill, split or crush them before distribution, which practice almost completely destroys their capacity for germination [see Greig, 1917]. Husking of lentils greatly improves their appearance, as the removal of the brown skin leaves a product orange pink in colour and of appetising appearance.

The guinea-pigs used were young growing animals of about 320 to 350 g. weight. They were mostly bred in the Department, to obtain a regular standard of health and vigour.

III. EXPERIMENTS SHOWING THAT DRY PEAS HAVE SMALL ANTI-SCORBUTIC VALUE.

In our experience, young guinea-pigs cannot be induced to consume any considerable amount of either peas or lentils in the dried state. An attempt was made to overcome this difficulty by offering it in the form of meal. The dry peas were ground in a coffee mill and the resulting meal sifted free from the finer particles (which are apt to cake together in a sticky mass when wetted) and shaken free of the skins. The coarse meal thus obtained was moistened with water and allowed to stand for half an hour before being offered to the animals. It was not eaten rapidly, so that some, at least, of the meal was in the soaked condition for some hours before being actually consumed. Residues left over from a day's ration were washed free from particles of the peat on which the animals were bedded, air-dried and weighed.

Three animals were placed upon a diet of oats and bran *ad lib.*, autoclaved milk to 60 cc. and 15 g. dried ground peas (Table I). In no case was this ration of pea-meal eaten regularly and the average amount consumed daily varied from 7 to 10 g. One animal (349) died after 31 days with every symptom of acute scurvy. The other two (347, 348) developed scurvy which became chronic. No. 348 (*B*, fig. 1) consumed a daily average of 9.6 g. pea-meal; after 44 days it became lame, developed loose teeth and had to be fed daily by hand with milk and little pellets of the moistened pea-meal. It continued for a long time in this condition and when killed by chloroform at the end of 105 days was found to be fairly well nourished. There were, however,

Table I. *Experiment with dry peas.*

Number of animal	Ration offered	Average amount consumed daily			Body weight g.	Symptoms during life	Duration of experiment, days	Post-mortem	Histology of rib-joints	General result
		Peas g.	Oats and bran g.	Milk cc.	Initial	Final				
348	15 g. dry pea-meal	9.6	17	52	325	410	105	Bones brittle, rib junctions enlarged, extensive old and recent haemorrhages	Scurvy	Chronic scurvy
349	" "	8.8	20	37	340	315	21	Bones fragile, rib junctions enlarged, muscular and visceral haemorrhages	Severe scurvy	Died of severe scurvy
347	" "	8*	20	40	350	345	30 } 59 } 101 } 15 }	Bones brittle, rib junctions ridged, haemorrhages not severe	? Scurvy†	"

Table II. *Experiments with soaked peas.*

Number of animal	Ration offered	Average amount consumed daily			Body weight g.	Symptoms during life	Duration of experiment, days	Post-mortem	Histology of rib-joints	General result
		Peas g.	Oats and bran g.	Milk cc.	Initial	Final				
212	30-40 g. soaked peas	30-40	25 (for 38 days)	30-100 (from 38th day)	375	—	130	—	—	Good health, returned to stock
211	30-40 g. "	28	8	26	310	Slight soreness	128	Bones slightly brittle, rib junctions enlarged, slight haemorrhages, loose teeth	Scurvy	Killed: poor health; slight signs of scurvy
215	30-40 g. "	30	24	0	320	Limbs tender	40	Slight haemorrhage in heart muscle, teeth slightly brittle	? Scurvy	Death from unknown cause
88	20 g. "	28	32	0	320	Slight soreness of joints	34	Bones fragile, rib junctions ridged, severe haemorrhages	—	Death from severe scurvy
87	20 g. "	19	20	33 (last 32 days)	340	Slight soreness of limbs	42	Bones and teeth somewhat brittle	Scurvy	Poor health
208	40 g. "	17.5	51	0	500	Slight soreness	46	Ribs ridged, teeth loose, haemorrhages	—	Death from scurvy
213	30 g. "	18	24	0	358	Slight soreness	67	Bones normal, incisors loose, haemorrhages on cecum	? Scurvy	Death from scurvy
91	10 g. "	10	30	0	350	Soreness of limbs, loose teeth	21	Bones fragile, severe haemorrhages	—	Death from severe scurvy
92	10 g. "	10	28	0	380	do.	28	Bones fragile, severe haemorrhages	Scurvy	Death from severe scurvy
89	20 g. soaked peas replaced after 36 days by germinated peas	20	33	50 (last 48 days)	330 } 335 }	Soreness of joints	38 } 76 }	Normal	Normal	Scurvy cured by germinated peas
90	10 g. soaked peas replaced after 24 days by 30 g. germinated peas	10	29	55 (last 75 days)	335 } 335 }	Soreness of limbs, loose teeth	24 } 84 }	—	—	Returned to stock in good health; scurvy cured by germinated peas

* For 30 days, pea-meal then replaced by tamarind juice for 59 days.

† Here and in the other tables "? scurvy" indicates that the rib junctions were not normal, and in most cases they showed the changes which have been previously described as "incipient scurvy" (Tozer, 1918); as there mentioned, however, it is doubtful whether these alterations indicate anything more than some imperfection in the supply of the necessary substances concerned with growth. They cannot be taken as necessarily indicating scurvy, but they are certainly compatible with early or mild scurvy and have to be considered along with the symptoms and other post-mortem signs in estimating the effect of scorbutic diets.

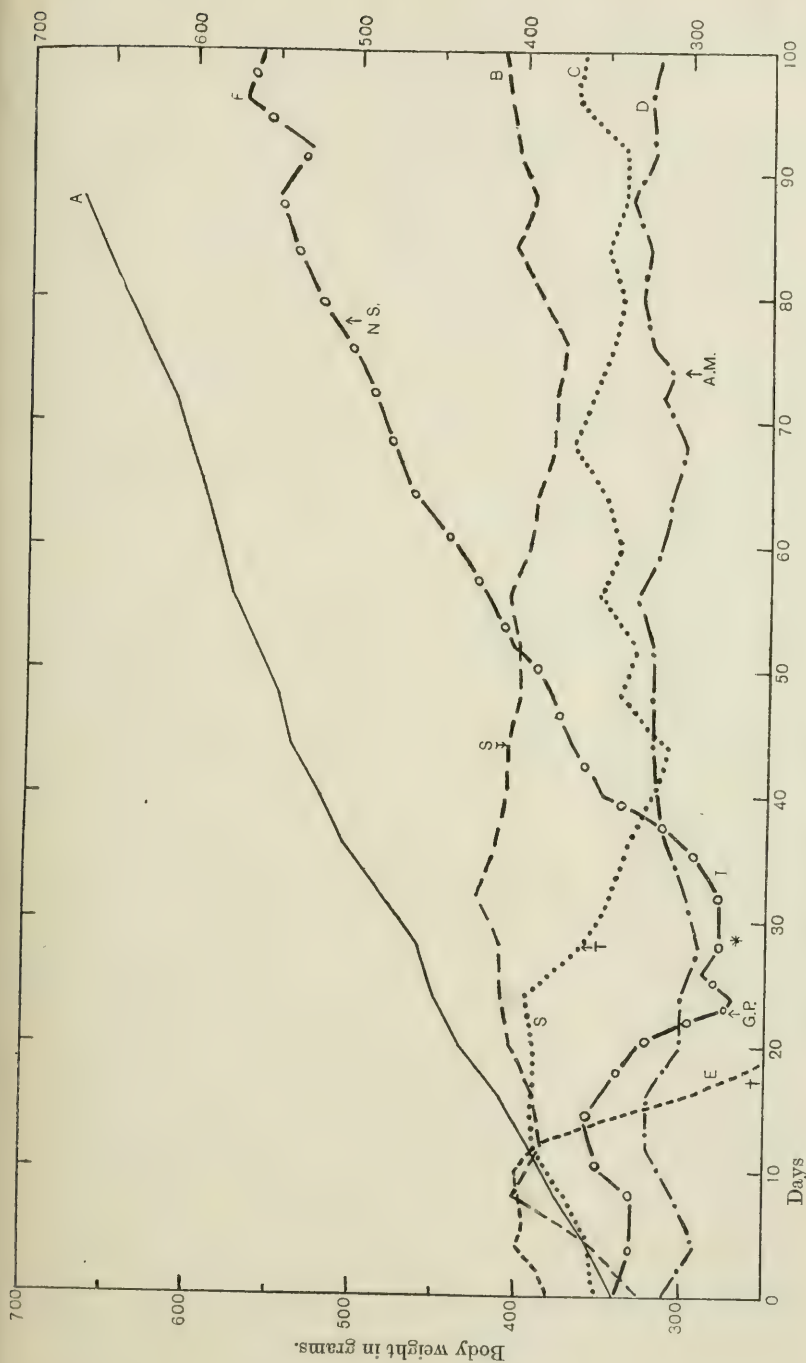


Fig. 1. Weight curves of animals on a diet of ungerminated soaked peas.

A. Control normal weight curve on 30 g. fresh cabbage, oats, bran, and water. B. Curve of No. 348 on average daily ration of 9.6 g. pea-meal, 52 cc. autoclaved milk, and oats and bran *ad libitum*. (Tab. I.) Symptoms of severe scurvy were first noted at S. C. Curve of No. 347 on average daily ration of 8 g. pea-meal, 40 cc. autoclaved milk, oats and bran *ad libitum* for first 30 days. Symptoms of scurvy were first seen at S; after 30 days the pea-meal was replaced by tamarind juice and after 89 days both pea-meal and tamarind juice were given daily. D. Curve of No. 214 on an average daily ration of 28 g. soaked peas: at A.M. after 74 days, an average of 26 cc. autoclaved milk was taken daily. E. Curve of No. 91 on an average daily ration of 10 g. soaked peas (died with severe symptoms of severe scurvy). F. Curve of No. 90 on an average daily ration of 10 g. soaked peas. After 24 days (at G.P.) the animal showed all the symptoms of severe scurvy, and the soaked peas were replaced by 10 g. germinated peas, and after 28 days (at *) the ration was increased to 30 g. After 34 days (at I) there was marked improvement, and 60 cc. autoclaved milk was added to the diet: growth at once began and after 78 days (at N.S.) no further symptoms of scurvy could be detected.

extensive haemorrhages in the muscles both recent and of old standing, the bones were fragile and the rib junctions deformed. No. 347 (C, fig. 1) developed swollen and painful joints after 21 days and after 30 days was very lame. Its ration of pea-meal, of which it had eaten about 8 g. daily, was then discontinued and its place taken by a daily ration of 15 to 20 cc. of the juice expressed from dried tamarind, previously swelled in water; the rest of the diet remained as before. It lived 59 days on the tamarind juice without showing much change, further progress of the disease being arrested. It was then given pea-meal in addition, no improvement resulted and the animal was chloroformed after a further period of 15 days. The subsequent post-mortem and histological examinations indicated a condition of chronic scurvy.

It is evident from these results that some small degree of protection from scurvy may be derived from a daily dose of 8 to 10 g. of moistened pea-meal. In comparison with other anti-scorbutic materials it has, however, very little potency. Taking, as is our custom, fresh cabbage leaves as the standard, Delf [1918] has shown that young guinea-pigs can be protected from scurvy and maintained in satisfactory health on a diet of oats, bran, autoclaved milk and 1.5 g. fresh cabbage daily: with 0.5 g. definite symptoms of scurvy appear, varying in intensity in different guinea-pigs. It is evident, therefore, that an average daily ration of 9 g. pea-meal has a protective value less than that of 1.5 g. and about equivalent to that of 0.5 g. fresh cabbage: in other words, cabbage is about 15 times as effective as dry peas. The water content of dry peas is about 12 %, of cabbage about 90 %, so that on the dry weights 8 g. of peas are about equal to 0.05 g. cabbage, a ratio of 160 to 1.

IV. EXPERIMENTS SHOWING THAT PEAS AND LENTILS, AFTER SOAKING IN WATER FOR 24 HOURS, HAVE LITTLE ANTI-SCORBUTIC VALUE.

During the process of soaking, the peas and lentils absorbed roughly their own weight of water and the soaked seeds contained 50 to 55 % water, estimated by drying at 100 to 110°. It was difficult to coax the animals to eat large rations; in one case (No. 212), 30 to 40 g. daily were taken; in general, however, 15 to 20 g. soaked peas and 10 to 15 g. lentils was the limit that was voluntarily consumed. Lentils were eaten without preparation, but it was necessary to skin the peas before the guinea-pigs would eat them; this was done after the ration had been weighed out and involved a loss of about 10 % in weight.

In Table II are given the results of experiments with dry green peas after soaking 24 hours in water at room temperature. Scurvy was prevented when a daily ration of 30 to 40 g. was consumed; with 15 to 30 g., scurvy occurred and although two animals out of six survived the three months of the experiment, they had poor health and made little or no growth. When the ration was reduced to 10 g. death from scurvy occurred as in control animals

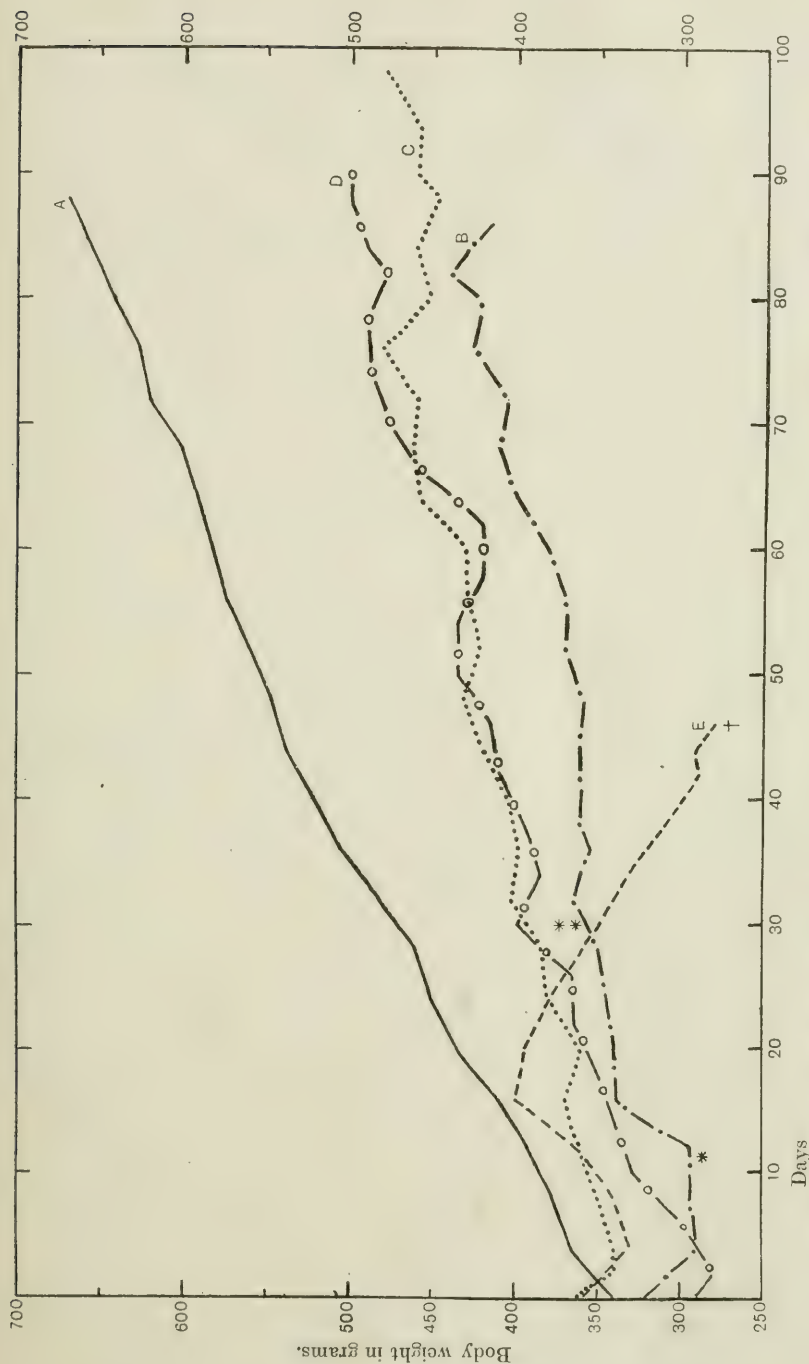


Fig. 2. Weight curves of animals on a diet of ungerminated soaked lentils with oats and bran *ad libitum* in each case. A. Control normal weight curve on 30 g. raw cabbage, oats, bran and water. B. No. 409, on an average ration of 25 g. soaked lentils daily. At * 30 cc., and at † 60 cc. autoclaved milk was added to the diet. C. No. 415 on an average ration of 18 g. soaked lentils. From the first, 30 cc. and after 12 days 60 cc. autoclaved milk was included in the diet. Symptoms of scurvy were first observed after 68 days. D. No. 412 on an average ration of 15 g. soaked lentils. For the first 20 days 30 cc. and afterwards 60 cc. autoclaved milk was given daily. E. No. 413 on an average ration of 10 g. soaked lentils daily. After 4 days 30 cc. and after 26 days 60 cc. autoclaved milk was added to the diet.

(*e.g.* 91 and 92, which died from scurvy in 21 and 28 days). About half the animals received no milk, but the result as regards scurvy was not apparently affected by this.

The case of 89 is interesting. Scurvy, developing on a 20 g. ration of soaked peas, was cured when this ration was substituted by an equivalent weight of germinated peas. Guinea-pig 90 was cured in the same way.

These experiments afford no evidence that soaked peas are better than dry peas. The dry substance of 9 g. dry peas is about equal to that of 18 g. soaked peas, and the effect of the two rations is not markedly different.

Soaked lentils (Table III) have an anti-scorbutic value which is slightly greater than that of soaked peas. Scurvy was prevented with a daily consumption of about 25 g.; with 15 to 18 g. scurvy was apparent but not severe, and 412 and 415 survived the three months of the experiment and put on weight. On a daily consumption of about 9 g., death from severe scurvy occurred in 45 days with much loss of weight. All the animals received autoclaved milk.

In comparing the value of peas and lentils, it should be noted that 10 g. of peas have an average of 30 seeds, and 10 g. of lentils have about 100 seeds.

V. EXPERIMENTS SHOWING THAT GERMINATED PEAS AND LENTILS POSSESS CONSIDERABLE ANTI-SCORBUTIC VALUE.

The peas and lentils after soaking in water for 24 hours were kept moist with access of air (conveniently in a large funnel) for about 48 hours at room temperature. The radicle was then usually about 1 cm. in length [see the figures in the paper by Chick and Hume, 1917]. The guinea-pigs ate the germinated seeds more readily than when they were merely soaked, though they seemed unable to take more than about 15 g. of lentils daily, whether raw or cooked: with peas, 20 to 30 g. were eaten daily for two or three months without ill effect.

Twenty-four animals received varying amounts of germinated peas (Table V). With a daily consumption of 10 g. and over there was satisfactory protection except in the case of one animal (315) out of ten. Out of six animals on a 5 g. ration, two died of some infection: four lived through the three months of the experiment; these grew fairly well and clearly received considerable protection, although they showed some evidence of scurvy when they were killed for examination. On 2.5 g. two died of acute scurvy in 37 and 53 days respectively and though the other two survived to the end of the experiment, both had the disease in a moderate degree. Four animals which ate daily 7 to 10 g. of peas germinated in the dark were also well protected.

With regard to the growth of these animals, it should be noted that most of those which had the larger pea rations were started with peas, oats and bran, but without milk. On this diet little growth was made. The addition

of autoclaved milk at once initiated growth (312, Fig. 3, *B*): autoclaved skimmed milk acted in the same way though less effectively (308, Fig. 3, *C*): with tinned milk (315, Fig. 3, *D*) there was a short period of improvement but the animal afterwards lost weight, refused food and died with diarrhoea.

These scanty data suggest that germinated peas are deficient in the fat-soluble growth factor of which whole milk seems to be the best source among

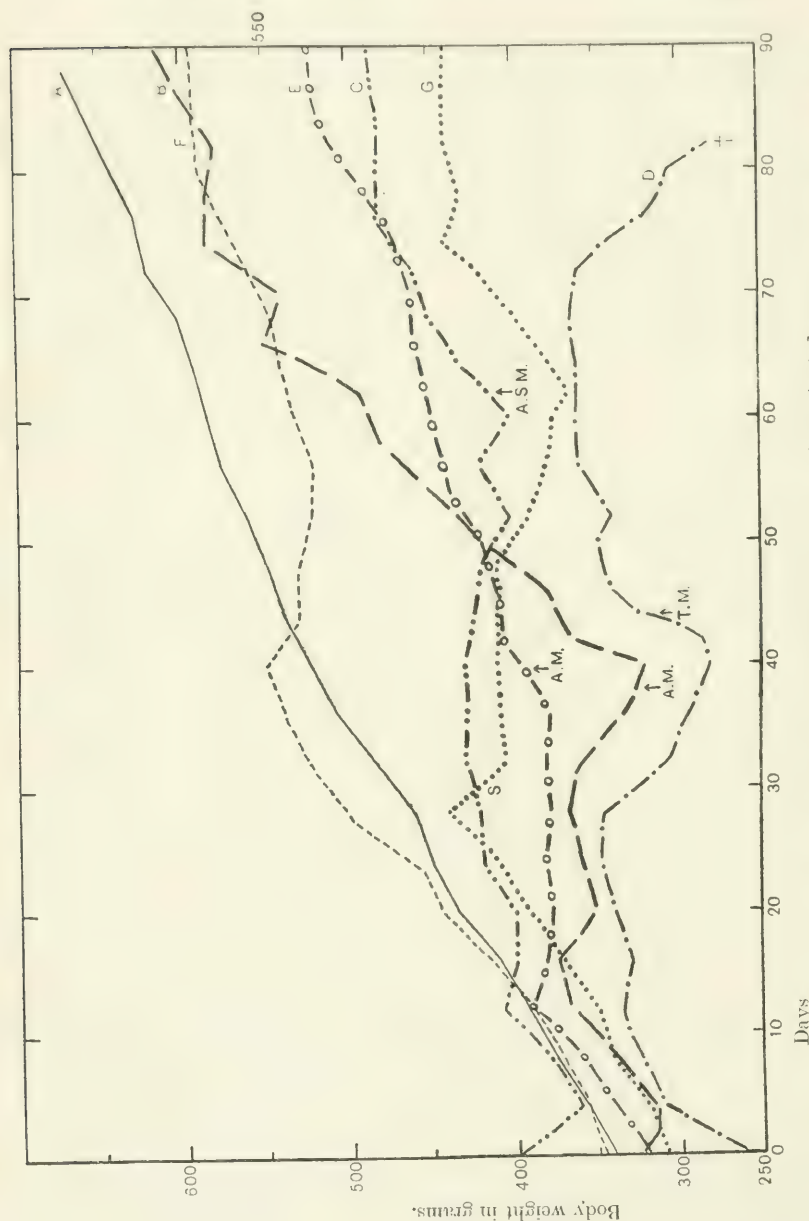


Fig. 3. Weight curves of animals on a diet of germinated peas.

A. Normal weight curve on 30 g. fresh cabbage, oats, bran and water. *B.* No. 312 on an average daily ration of 20-30 g. germinated peas. After 38 days (at *A.M.*) 60 cc. autoclaved milk was added to the diet. *C.* No. 308 on a daily ration of 20 g. germinated peas. After 62 days (at *A.S.M.*) autoclaved skimmed milk was added to the diet. *D.* No. 315 on an average daily ration of 30 to 12 g. germinating peas. After 44 days (at *T.M.*) tinned milk was added to the diet, but the appetite for peas diminished to about 2 g. daily, and scurvy symptoms appeared. *E.* No. 310 on average daily ration of 10 g. germinated peas. After 38 days (at *A.M.*) autoclaved milk was added to the diet. *F.* No. 337 on a daily ration of 5 g. germinated peas with 60 cc. autoclaved milk from the first. *G.* No. 437 on a daily ration of 2.5 g. germinated peas with 60 cc. autoclaved milk from the first. After 30 days, at *S.* symptoms of scurvy first developed.

the different types of milk tried. Before this conclusion could be accepted as an established fact, it would be necessary to show that the diet was adequate as regards all *other* components, to a deficiency of which growth limitation could be attributed—such as the nature and quantity of (1) protein, (2) mineral salts, and (3) water-soluble growth factor. No. 3 is provided in abundance in a diet consisting of peas, oats, and wheaten bran, and it is probable that a possible defect in any of these constituents as regards (1) or (2) will be made good by admixture with the others. The fact that in all these experiments without milk, weight was satisfactorily *maintained* is in favour of this conclusion. It is unfortunate that the point could not be quickly settled by observing whether normal growth takes place on addition to the diet of a small proportion of butter fat, cod liver oil, or other source of the fat-soluble growth factor—the guinea-pig being unable to tolerate pure fat given in this form.

In case of the animals with the smaller rations of peas, 60 cc. of autoclaved milk was offered daily throughout the experiment and was as a rule well taken; in the absence of severe scurvy growth was made (Fig. 3, compare *F* and *G*). It seems, therefore, that a daily ration of 10 g. germinated peas with milk will prevent scurvy and assure good health and growth. The cure of two animals from scurvy by germinated peas has already been mentioned (see Table II).

With germinated lentils (Table IV) complete protection was obtained with a daily consumption of about 10 g. and partial protection with 5 g., while with 2.5 g. definite symptoms developed in every case. In all cases life was substantially prolonged, even when scurvy was not prevented.

With 10 g. of lentils no growth was made without milk but when autoclaved milk was added to the oats and bran, the weight rapidly increased (Fig. 4, *B*, after 30 days). The 2.5 and 5 g. series had milk from the start and made good growth until symptoms of scurvy appeared. The history of the guinea-pigs with the smallest lentil rations was, however, unfortunately obscured by four of the six animals dying of intercurrent disease as is often the case when the diet contains only minimal amounts of an essential accessory factor.

The results with germinated lentils are thus in accord with those for peas: in both cases a 5 g. daily ration seems to be just below, and 10 g. well above, the minimum required for protection.

In Table VI are summarised the results of the foregoing experiments with peas and lentils. In the dry condition peas show traces of anti-scorbutic properties as evidenced by prolongation of life although scurvy is not prevented; the same result was obtained by Fürst [1912]. When soaked in water for 24 hours at room temperature scurvy was prevented with a large daily ration, 30 to 40 g. peas (dry weight, 15 to 20 g.) or 20 to 30 g. lentils (dry weight, 10 to 15 g.). The data do not permit one to decide whether any anti-scorbutic virtue is created during the process of soaking in water, for in the case of the

Table V. *Experiment with germinated peas.*

Number of animal	Ration offered	Average amount consumed daily			Body weight		Symptoms during life	Duration of experiment, days	Post-mortem	History of rib junctions	General result
		Peas g.	Oats and bran g.	Milk cc.	Initial	Final					
312	30 g. germinated peas	25	28	50 (after 36 days)	330	620	All the animals on 10 g. peas and upwards showed signs of soreness of limbs during life unaccompanied, however, by symptoms of scurvy, excepting in one case (No. 315)	93	Normal	Normal	Protection, no scurvy
323	30 g.	23	25	40 (after 26 days)	410	51		83	—	—	Good health, returned to stock do.
314	30 g.	21	25	40 (after 29 days)	270	515		88	—	—	do.
315	30 g.	30 to 12	24	47 (after 44 days)	280	270		75	Fragile bones, loose teeth, muscular and intestinal haemorrhages	—	Death from diarrhoea, scurvy also
308	20 g.	20	28	50 (after 60 days)	400	495		93	—	—	Good health, returned to stock
309	20 g.	18	29	52 (after 38 days)	305	550		93	Normal	Normal	Protection, no scurvy
325	15 g.	14.5	25	48	420	600		84	—	—	Good health, returned to stock
311	10 g.	10	28	52	320	515		94	Rib junctions slightly ridged	Normal	Protection, no scurvy
313	10 g.	10	30	47	290	392		69	Bones somewhat fragile, haemorrhages only in ileum and duodenum	Normal	Died from unknown cause, ? any scurvy
310	10 g.	10	27	52 (after 38 days)	320	535		93	Rib junctions slightly ridged	? Scurvy	Protection
327	5 g.	5	37	53	345	525	Slight soreness persistent	94	Rib junctions slightly thickened	Severe scurvy	Scurvy
328	5 g.	4.1	25 (after 68 days)	39 (after 68 days)	305	435	Slight soreness persistent	87	Rib junctions slightly ridged, muscles very dark	? Scurvy	Protection
337	5 g.	5	26	45	345	560	Scurvy position	97	Rib junctions slightly enlarged, slight haemorrhages do.	Scurvy	Slight scurvy
341	5 g.	5	28	44	350	565	Slight soreness	92	Slight subcutaneous haemorrhage	Normal	Slight scurvy
434	5 g.	5	29	45	330	360	None	29	—	Severe scurvy	Death from infection, also scurvy do.
435	5 g.	5	47	55	330	378	Soreness persistent	62	Spleen diseased	do.	do.
436	2.5 g.	2.5	34	46	343	272	Loss in weight	53	Bones very fragile, severe haemorrhages	do.	Death from severe scurvy
437	2.5 g.	2.5	35	59	309	433	Joints swollen and tender	94	Muscular haemorrhages partly absorbed	Scurvy	Chronic scurvy
438	2.5 g.	2.3	22	51	330	250	Soreness, scurvy position	37	Bones very fragile, teeth loose and brittle, haemorrhages	Scurvy	Death from severe scurvy
439	2.5 g.	2.5	46	56	325	645	Soreness lasting 32 days	90	Rib junctions slightly enlarged, teeth loose, jaw brittle	? Scurvy	Slight scurvy
342	10 g. peas germinated in darkness	7	22	57	328	515	Slight soreness	94	Normal	Normal	Protection
343	10 g. do.	10	24	52	340	642	Slight soreness lasting 35 days	102	—	—	Good health, returned to stock
344	10 g. do.	9.5	21	51	313	480	Scurvy position	94	No evidence of scurvy do.	Normal	Protection
345	10 g. do.	9	28	49	340	450	None	58	—	? Scurvy	Death from intestinal obstruction

— = not examined.

pea-meal it was not possible to induce the animals to take a ration larger than 10 g. (dry weight, 9 g.), but the change, if any, cannot be large.

After germination, however, the story is quite otherwise; scurvy was prevented successfully in case of both lentils and peas with a daily ration of 10 g. (dry weight, 5 g.); in many cases 5 g. daily (dry weight, 2.5 g.) was found to be sufficient, while with 2.5 g. daily (dry weight, 1.2 g.) some degree of protection was obtained.

Table VI.

Anti-scorbutic material	(Daily ration, g.	Water content, % (approx.)	Dry weight, g.	No. of animals	Time of experiment, days	Result
Peas						
Pea-meal, moistened	8-10	12	7-8.8	3	31-105	Scurvy, but some degree of protection
Soaked 24 hours	... 30-40	50	15-20	2*	128-130	No scurvy
	20	50	10	4	34-114	Scurvy, but distinct protection
	10	50	5	2	21-28	Death from scurvy
Germinated 20-30	50	10-15	6	75-93	Good health in 5 cases
	10	50	5	3	69-94	No scurvy
	5	50	2.5	5†	87-97	Signs of scurvy, but good health in 4 cases
	2.5	50	1.2	4	37-94	Scurvy in all cases, but some degree of protection
Lentils						
Soaked 24 hours	... 30	50	15	2	54-87	No scurvy, protection
	20	50	10	2	90-94	Signs of scurvy but considerable protection
	10	50	5	2	45	Death from scurvy
Germinated 10	50	5	4	91-94	No scurvy, protection
	5	50	2.5	4	51-90	Scurvy in 2 cases, but considerable degree of protection
	2.5	50	1.2	6	48-90	Scurvy, but considerable protection
Fresh lemon juice ...	1.5 cc.	—	—	6	74-90	No scurvy, protection
Fresh orange juice ...	1.5 cc.	—	—	5	62-91	" "
Fresh cabbage leaves	1.5 g.	90	—	5	90	" "
Fresh swede juice ...	2.5 cc.	—	—	8	36-90	" "
Fresh carrot juice ...	20-30 cc.	—	—	8	27-94	" "
Fresh beetroot juice ...	20 cc.	—	—	3	47-90	Scurvy
Fresh green beans ...	5 g.	90	—	4	—	Protection, no scurvy
Control A† ...	—	—	—	4	23-30	Death from scurvy
Control B† ...	—	—	—	4	34-40	" "

* Omitting No. 215 which died from other causes.

† Omitting 434 and 435 which suffered from an infection.

‡ In case of A the diet consisted of oats, bran and water only; in case of B autoclaved milk 60 cc. was added to the diet.

The anti-scorbutic value of the soaked seeds is thus seen to be increased 3- to 6-fold after 48 hours of germination at room temperature and becomes comparable with that of many fresh vegetables. While distinctly less than that of raw cabbage (minimum protective dose for guinea-pigs 1.5 g. [Delf, 1918]), swede (minimum protective ration 2.5 g. [Chick and Rhodes, 1918]), or fresh orange or lemon juice (minimum protective dose 1.5 cc. [see Chick, Hume, Skelton and Smith 1918]), it is equal to that of raw green (runner)

beans (minimum dose 5 g., unpublished results, Chick and Campbell) and superior to that of raw carrot (minimum dose 20 to 30 g.) or raw beetroot (minimum protective dose more than 20 g. [see Chick and Rhodes 1918]).

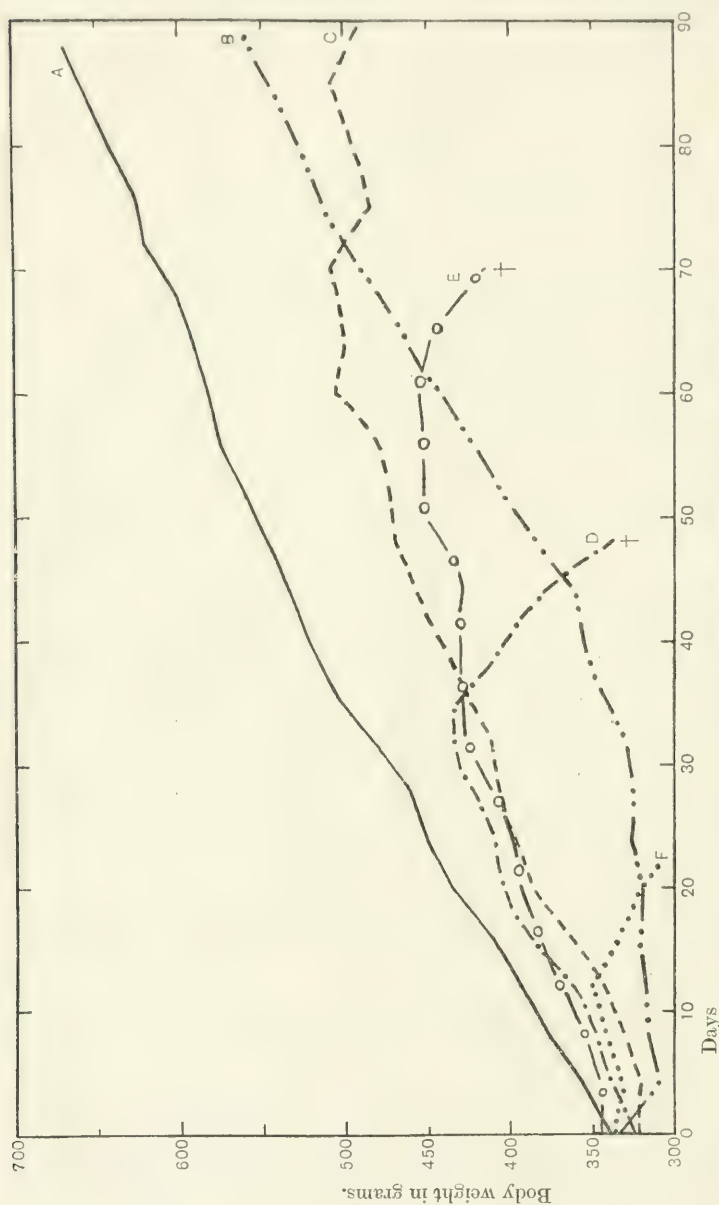


Fig. 4. Weight curves of animals on a diet of raw germinated lentils, oats, bran, and autoclaved milk.

A. Normal weight curve on 30 g. raw cabbage, oats, bran and water. B. Mean weight curve of four animals (Nos. 404, 405, 407, 411, Tab. IV) on 10 g. germinated lentils. C. Mean weight curve of 427, 428 A, the two best animals on 5 g. germinated lentils and 60 cc. autoclaved milk daily. No symptoms of scurvy were seen during life. D. Mean weight curve of 829, 830, the remaining two animals on 5 g. germinated lentils and 60 cc. autoclaved milk daily. E. Mean weight curve of Nos. 429, 430, 431, 817, 839, on 2.5 g. raw germinated lentils. At † No. 431 died. F. Mean weight curve of Nos. 819, 820, 821 (Tab. VII) on ration of 10 g. germinated lentils boiled in water half an hour, and 60 cc. autoclaved milk.

VI. EXPERIMENTS WITH COOKED GERMINATED LENTILS.

Animals were selected which ate raw lentils with relish and amongst these a further selection was made of those which would eat them readily after cooking. It was found (Table VII) that a ration of 10 g. boiled for half or one hour in water was insufficient to protect from severe scurvy. The animals did well for the first two weeks, they put on weight (Fig. 5, *D*), but soon suffered from swollen and sore joints and, when chloroformed, were all found to be suffering from severe scurvy. These animals all received a milk ration.

Five animals were next given a larger ration, 15 g., boiled in water for only 15 minutes, but it was found impossible to ensure that the whole of the amount was eaten. In all cases life was prolonged. One animal, 852 (Fig. 5, *C*), ate an average of 14 g. daily for 91 days and received considerable protection, but even this case showed swollen knees and lameness during life. The remaining four animals were all severely crippled after 30 to 40 days. Cooking had, therefore, reduced the anti-scorbutic value of about 12 g. of germinated lentils to rather less than that of 2.5 g. of the raw material (see Table IV), *i.e.* a loss of about 75 %. This result is in accord with that already established in the case of fresh cabbage, which has been shown to lose about 70 % of its antiscorbutic value when steamed for 20 minutes at 100° [Delf, 1918].

Major E. D. W. Greig, I.M.S., has suggested that the loss during boiling could be reduced by adding traces of some acid to the cooking water. Small amounts of citric acid have no harmful effects on young guinea-pigs [Delf, 1918]. With cabbage, citric acid accelerates the destruction of antiscorbutic stuff by boiling, and with germinated lentils very similar results have been obtained. The cell-sap of the growing lentils is slightly alkaline to litmus and remains neutral or slightly alkaline after boiling with a 0.005 % solution of citric acid. When the concentration is increased to 0.5 %, however, the washed seeds yield a juice which is distinctly acid to litmus. These two solutions were used respectively as the cooking water for the rations in two sets of experiments (Table VIII), and the results show that there is certainly no advantage, and possibly some disadvantage, in using acidified instead of ordinary tap water.

VII. WILTSHIRE'S EXPERIMENTS ON THE VALUE OF GERMINATED BEANS IN THE TREATMENT OF HUMAN SCURVY.

Acting on the suggestion made in the preliminary statement of these results [Chick and Hume, 1917, p. 157], Major H. W. Wiltshire, R.A.M.C., has made a most interesting trial of germinated beans in the treatment of scurvy in Serbian soldiers [1918]. Peas could not be obtained, the available lentils were decorticated and would not germinate, so "haricot beans were...first soaked in clean water for 24 hours, and then placed in tin trays for 48 hours to germinate. Old ration biscuit tins, cut in half longitudinally

Table VII. *Experiment with cooked germinated lentils.*

Number of animal	Ration offered	Average amount consumed daily		Body weight g.	Symptoms during life	Duration of experiment, days	Post-mortem	Histology of rib junctions	Clinical history
		Lentils g.	Oats and bran g.						
809	10 g. germ. lentils boiled 1 hr. in water	9.4	28	Initial 320 Final 260	Scurvy position, great soreness	25	Bones fragile, haemorrhages	Severe scurvy	Severe scurvy
810	do.	8.3	10	325	do.	30	"	"	"
811	do.	10.3	15	350	Scurvy position, lame	30	"	"	"
812	do.	8.2	14	322	do.	29	"	"	"
819	10 g. germ. lentils boiled 1 hr. in water	9.2	13	340	Soreness, scurvy position	21	"	"	"
820	do.	6.6	15	335	do.	26	"	"	"
821	do.	8.5	13	340	do.	37	"	"	"
822	do.	10.2	17	325	do.	27	Not examined	Not examined	Severe scurvy, cured with orange juice
846	15 g. germ. lentils boiled 15 min. in water	12.3	12	370	Sore and much swollen knees, scurvy position	82	Bones fragile, severe haemorrhages	Severe scurvy	Emaciated, lame, partial prostration
849	do.	12.1	10	325	do.	53	do.	do.	Death from severe scurvy
850	do.	6.3	11	325	do.	52	do.	do.	"
851	do.	13.7	13	350	do.	60	Bones firm, old haemorrhages at knees, slight recent haemorrhage	Severe scurvy	"
852	do.	14.2	22	360	do.	91	"	? Scurvy	Partial prostration

Table VIII. *Experiment with germinated lentils cooked with citric acid.*

859	15 g. germ. lentils boiled 15 min. in 0.005 % citric acid	12.9	15	325	Soreness, knees swollen	94	Bones normal, slight muscular haemorrhages about knees	? Scurvy	Slight scurvy
860	do.	13.3	8.5	330	do.	60	Bones soft, loose teeth, severe haemorrhages	Severe scurvy	Death from severe scurvy
872	do.	12.5	9	352	None	38	Bones slightly fragile, ribs slightly ridged	Scurvy	Death from citric acid alone
873	do.	9.4	6	331	Knees swollen	36	Bones papery, loose teeth, severe haemorrhages and in one lung	Severe scurvy	Death from severe scurvy
863	15 g. germ. lentils boiled 15 min. in 0.5 % citric acid	11.7	9	337	Scurvy position	30	Bones fragile, haemorrhages severe	"	"
864	do.	12.9	9	333	Sore, knees swollen, scurvy position	89	Bones fragile, rib-junctions nodular, severe haemorrhages	Severe scurvy	Chronic scurvy
874	do.	15	7	320	Sore, swollen knees	90	Bones fragile, rib-junctions ridged, two ribs broken, severe haemorrhages	"	"
875	do.	13.3	5	335	Sore, swollen knees, became helpless	59	Bones very fragile, rib junctions much swollen, severe haemorrhages	"	Death from severe scurvy

and freely perforated with holes, were found serviceable for this purpose. They were easy to make, clean to handle, and each half held seven pounds of beans, a day's dose for 28 patients. Since germination takes about 48 hours at 60° F., it can easily be carried out in this country (Serbia) in May, when the mean temperature is 67° F. The whole process is very simple, the only essentials being that the seeds must be kept moist but not shut off from

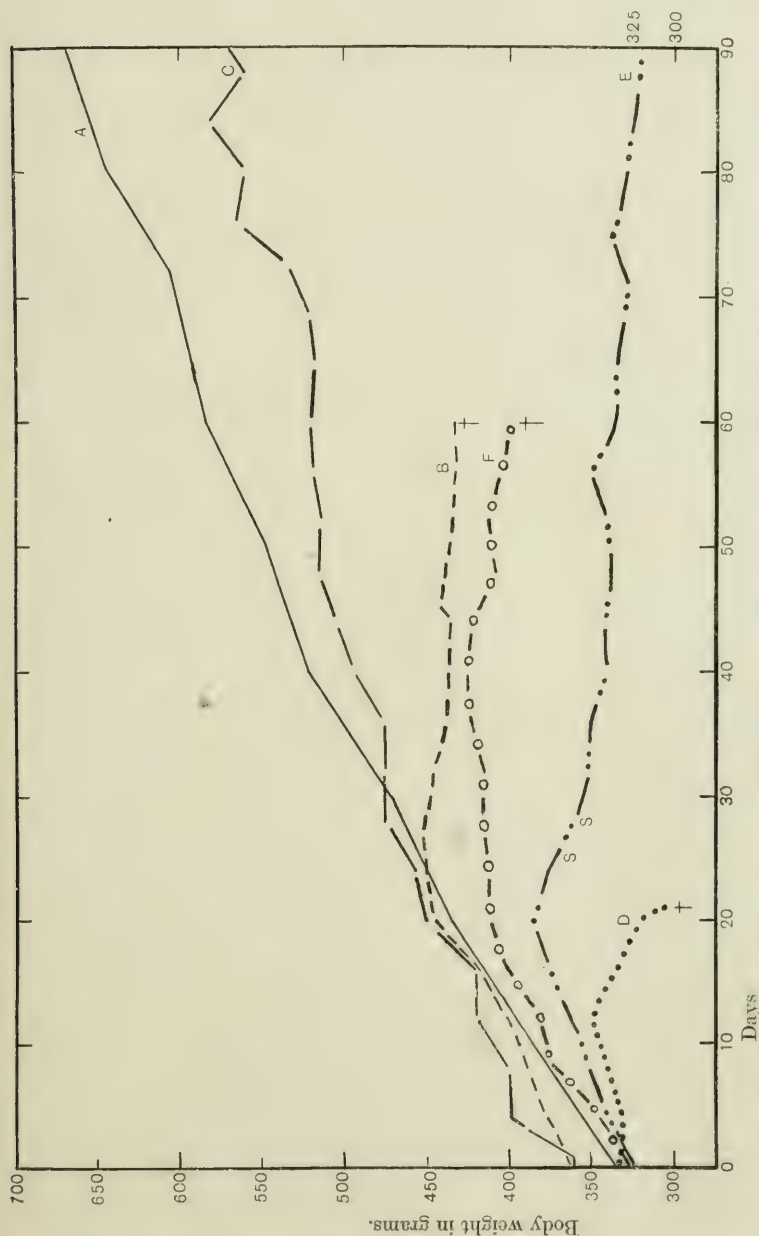


Fig. 5. Weight curves of animals on a diet of cooked germinated lentils, oats, bran and autoclaved milk. A. Control normal weight curve on 30 g. raw cabbage, oats, bran and water. B. Mean weight curve of 5 animals on diet of 10-15 g. germinated lentils boiled 15 min. in water, oats, bran and 60 cc. autoclaved milk daily; at † No. 852 died. C. Weight curve of No. 852, the only animal of this set which escaped scurvy. D. Mean weight curve of three animals (819, 820, 821) on diet of 7-9 g. germinated lentils boiled half an hour in water, oats, bran and autoclaved milk. E. Mean weight curve of Nos. 864, 874, the two best cases on diet of 13-15 g. germinated lentils boiled 15 min. in solution of 0.5 % citric acid. At S, S, definite symptoms of scurvy were first seen. F. Mean weight curve of Nos. 859, 860, the two best cases on diet of 10-15 g. germinated lentils cooked 15 min. in solution of 0.005 % citric acid. At † No. 860 died.

free circulation of air. After germination the ten minutes' boiling was ample to fit the beans for eating.

"Two wards, each containing 27 beds, were specially devoted to the purpose of comparing the therapeutic values of these germinated beans and fresh lemon juice. Scurvy patients were admitted to these two wards alternately, without selection. In one ward each patient was given four ounces of fresh lemon juice daily, the juice being expressed in the ward, sweetened slightly and given as a measured dose by the sister. In the other ward each patient received a portion of germinated beans which had weighed four ounces in the dry state.

"Thirty patients were treated with lemon juice and twenty-seven with beans. Contrasting them as regards severity of disease at the commencement of treatment, those treated with beans were, on the whole, slightly worse. The difference would be too small to mention, save that it justifies the definite statement that the bean cases were certainly as severe as the lemon cases. Comparing the results of treatment there is a small but definite difference in favour of those treated with beans, 70 % being cured within four weeks as against 53 % of those treated with lemon juice. These figures favour the bean cases unduly, the real difference being better expressed by the (average) time taken for the gums to return to normal, which was 3.1 weeks for bean cases and 3.4 weeks for lemon cases."

Evidently germinated beans are satisfactory for treatment. Whether they are as efficient as fresh lemon juice cannot of course be gauged without further experience with different doses. Experiments on guinea-pigs show (Table VI above) that, as a preventive, lemon juice (but not lime juice: Chick, Hume, Skelton and Smith, *Lancet*, November 30th, 1918) is a good deal superior to germinated peas or lentils.

It is, however, as a *preventive* of scurvy that the inclusion of germinated pulses in a human diet deficient in fresh fruit and vegetables, is principally to be recommended, and so far no definite trials of this type have been reported. In many parts of the world there exists the practice of eating certain seeds in the germinated condition, although there is no suggestion that the anti-scorbutic value of such foods has been appreciated. In the Dutch Indies and Federated Malay States germinated beans or "tow-gay" are eaten raw as a common article of diet [Grijns, 1901; Private communication, Brig.-Gen. Anderson]. In certain districts of China it is the custom to take part of the rice in the germinated condition and especially in the north, beans are artificially sprouted for food in the winter [Report, 1885].

There is little doubt that the anti-scorbutic value of certain fermented beverages, firmly believed in by our ancestors, owed their virtue to the germinated grains from which they were prepared. Captain Cook¹ was a firm believer in the anti-scorbutic properties of a fresh infusion of malt, "sweet-wort." He always took large quantities of malt to sea with him and

¹ *Captain Cook's Voyages of Discovery.*

the sweet-wort was served out to his men in large quantities when there was any risk of scurvy. "Small beer" also enjoyed a great reputation and in many Arctic expeditions in the first half of the nineteenth century, malt, yeast, and a brewing plant were included in the equipment [see A. Henderson Smith, 1918].

Modern beer prepared from "high dried" malt has been shown by Harden and Zilva [1918] to possess no appreciable anti-scorbutic value and there is no doubt that the virtue these more ancient beverages possessed was due to the freshness of the product, the drink being prepared quickly from freshly germinated grain and consumed shortly after preparation. An interesting incident illustrating this point was reported during the present war by Dyke [1918], in connection with an outbreak of scurvy among a Kaffir labour battalion in France. The diet of this community was arranged to produce as nearly as possible that of the native kraals, where, in spite of a limited diet, scurvy is unknown. The arrangements even included the provision of a native beer brewed from millet, of which large quantities are regularly consumed in Africa. In the enquiry which followed the outbreak of scurvy, the following fact emerged, viz. that the beer as brewed in South Africa was prepared from germinated millet, but that in France the process of germination had been omitted for reasons of convenience.

SUMMARY.

1. Fürst's observation that the content of anti-scorbutic accessory factor in dry peas and lentils is much increased on germination has been confirmed.
2. The antiscorbutic value of these seeds after soaking in water for 24 hours, and germination for 48 hours at room temperature is 5- to 6-fold that of the dry seeds; while inferior to that of orange and lemon juice or cabbages and swedes, it is equal to that of many other vegetables such as green (runner) beans or potatoes, and superior to that of carrot or beetroot. Table VI contains a summary of these results.
3. These seeds, whether in the dry or germinated condition, do not, however, contain a sufficient amount of the growth-promoting substances to induce satisfactory growth in the experimental animals (guinea-pigs) in the absence of milk from the diet. It seems probable that it is the fat-soluble factor that is deficient.
4. A considerable proportion of the anti-scurvy power generated in these germinated seeds is destroyed by boiling: cooking of these germinated seeds should therefore be as short as possible.
5. Wiltshire has shown that germinated beans can be used with success in the treatment of human scurvy. Other evidence is quoted from experience of human diets showing that germinated seeds and their products form valuable material for the prevention of human scurvy.

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XXIII. THE EFFECT OF ALCOHOL ON THE DIGESTION OF FIBRIN AND CASEINOGEN BY TRYPSIN.

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THE behaviour of extracts of pancreas or pure pancreatic juice under different conditions has led various observers to conclude that the pancreas contains a number of proteolytic enzymes. It was shown by Fermi [1890] that after treatment with mercuric chloride, salicylic acid and various other substances, trypsin lost its power of digesting fibrin but would still digest gelatin. Vernon [1901] arguing from the varying sensitiveness of pancreatic extracts towards sodium carbonate, concluded that "trypsin" was really a mixture of enzymes of different degrees of stability, the more sensitive enzymes being destroyed first. Vernon only tested the digestive power of trypsin on raw fibrin in this connection however. In a later paper Vernon [1903, 2] states that pancreatic extracts contain an erepsin as well as trypsin. Pollak [1905] using different preparations of enzymes found that the relative amounts of serum and gelatin digested varied enormously in different cases. He also found that after treatment with hydrochloric acid trypsin lost its power of acting on serum, but was still about as active as ever on gelatin. Pollak concluded that extracts of pancreas contained in addition to trypsin (to which the action on serum was due) a special enzyme which acted only on gelatin. To this enzyme Pollak gave the name of glutinase.

According to Ascoli and Neppi [1908], however, this assumption of a special enzyme acting on gelatin is unjustified, as they find that slight variations in the reaction of the medium affect the digestion of different proteins to different degrees. Mays [1906] after a long series of experiments remarks that the presence of two proteolytic enzymes in pancreatic extracts can only be proved when it is possible to make a separation of the enzymes. It had been previously shown by Bayliss and Starling [1903] that pancreatic juice as secreted contains no trypsin (as tested on coagulated egg white), but contains a weak enzyme like erepsin. This has some action on caseinogen, but very slight. The erepsin has a slight action on fresh fibrin but practically none on fibrin which has been heated to 70°. It may here be mentioned that Long and Barton [1914] state that raw fibrin even when very carefully purified may soon become liquid owing to autolysis.

In later papers Fermi [1913, 1914] contests the theory that some proteolytic enzymes have a specific action, and maintains that all proteolytic enzymes have a general action on all proteins.

Slight differences of behaviour of trypsin towards different proteins under the same conditions have also been noted by Berg and Gies [1906], Porter [1910], Long and Hull [1917], but not much importance seems to have been attached to the facts. Others such as Glaessner and Stauber [1910] and Anerbach and Pick [1912] find differences between the proteolytic and peptolytic actions of trypsin, but in these cases possibly some of the action was due to the pancreatic erepsin also.

It seems to have been assumed, however, by all the authors quoted and by others such as Hedin [1905] that trypsin is the enzyme responsible for the digestion of fibrin and caseinogen, especially in experiments lasting only a few hours.

The action of alcohol on trypsin has been variously stated. Fermi and Pernossi [1891] using Mett's tubes filled with gelatin found that in presence of alcohol trypsin had more digestive action than in presence of water only. The percentage of alcohol used is not stated. Chittenden and Mendel [1896] found that the action of trypsin on fibrin was markedly inhibited by alcohol, but did not test the action on any other substrate. Dastre [1896] found that trypsin still digested fibrin and boiled albumin in presence of 15 to 20 per cent. of alcohol, while Gizelt [1906, 1, 2] states that 20 % alcohol totally inhibits trypsin. According to Bayliss [1915] trypsin will digest gliadin even in presence of 80 % alcohol, the action in this case being due to the trypsin in suspension. Vernon [1903, 1] noted that dilute alcohol had a considerable inhibitory effect on the digestion of raw fibrin by trypsin.

As dilute alcohol is frequently used in making extracts of various digestive organs, it is important to know how the digestive action is affected thereby.

EXPERIMENTAL DETAILS.

The experiments were carried out as described previously [Edie, 1914]. Ox fibrin after being finely minced and thoroughly washed was suspended in water and gradually heated to 85°. The fibrin was then pressed dry and preserved in glycerol and a little chloroform until required. The caseinogen was a 3 % solution in 1 % sodium carbonate. The pancreatic extracts were prepared by finely mincing sheep's pancreas and extracting with chloroform water for about a fortnight. The extract was then filtered and a little chloroform added as a preservative.

The digestion was carried on at 37° in small flasks, a small measured quantity of chloroform being added to exclude bacterial action in every case. When fibrin was used, the amount of digestion was estimated by filtering off the undissolved fibrin and determining the nitrogen in the filtrate by a Kjeldahl determination. When caseinogen was the substrate, the amount of

digestion was found by precipitation with tannic acid and subsequent estimation of the nitrogen in the filtrate. Controls showed that the sodium carbonate alone had no digestive action whatever either on fibrin or caseinogen. The following are typical results showing the effect of dilute alcohol on the digestion of fibrin and caseinogen by trypsin.

						Digestion in cc. of N/10 nitrogen
1.	(a)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. 1 % Na_2CO_3	4.6	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	17.1	
		1.4 g. fibrin added. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. caseinogen	23.8	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	23.6	
		Digestion 1 hour.				
2.	(a)	1 c.c. trypsin, 20 c.c. 12 % alcohol, 20 c.c. 1 % Na_2CO_3	4.6	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	13.8	
		1 g. fibrin. Digestion 2.75 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 12 % alcohol, 20 c.c. caseinogen	24.1	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	24.3	
		Digestion 1.25 hours.				
3.	(a)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. 1 % Na_2CO_3	3.8	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	14.1	
		1.3 g. fibrin. Digestion 2 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. caseinogen	27.9	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	27.0	
		Digestion 1.25 hours.				
4.	(a)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. 1 % Na_2CO_3	8.1	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	23.6	
		1.5 g. fibrin. Digestion 2.25 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 10 % alcohol, 20 c.c. caseinogen	30.0	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	29.3	
		Digestion 1 hour.				
5.	(a)	1 c.c. trypsin, 20 c.c. 8 % alcohol, 20 c.c. 1 % Na_2CO_3	5.1	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	12.1	
		1 g. fibrin. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 8 % alcohol, 20 c.c. caseinogen	30.6	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	30.6	
		Digestion 1.25 hours.				
6.	(a)	1 c.c. trypsin, 20 c.c. 8 % alcohol, 20 c.c. 1 % Na_2CO_3	4.9	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	12.1	
		1 g. fibrin. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 8 % alcohol, 20 c.c. caseinogen	27.7	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	28.0	
		Digestion 1 hour.				
7.	(a)	1 c.c. trypsin, 20 c.c. 6 % alcohol, 20 c.c. 1 % Na_2CO_3	11.5	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	20.3	
		1 g. fibrin. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 6 % alcohol, 20 c.c. caseinogen	22.2	
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	22.0	
		Digestion 1 hour.				

						Digestion in cc. of N/10 nitrogen
8.	(a)	1 c.c. trypsin, 20 c.c. 6 % alcohol,	20 c.c. 1 % Na_2CO_3	12.5
	(b)	1 c.c. trypsin, 20 c.c. water,	20 c.c. 1 % Na_2CO_3	22.2
		1 g. fibrin. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 6 % alcohol,	20 c.c. caseinogen	25.0
	(b)	1 c.c. trypsin, 20 c.c. water,	20 c.c. caseinogen	24.6
		Digestion 1 hour.				
9.	(a)	5 c.c. trypsin, 20 c.c. 16 % alcohol,	20 c.c. 1 % Na_2CO_3	7.1
	(b)	5 c.c. trypsin, 20 c.c. water,	20 c.c. 1 % Na_2CO_3	18.2
		1 g. fibrin. Digestion 2 hours.				
	(a)	5 c.c. trypsin, 20 c.c. 16 % alcohol,	20 c.c. caseinogen	31.6
	(b)	5 c.c. trypsin, 20 c.c. water,	20 c.c. caseinogen	31.9
		Digestion 1 hour.				
10.	(a)	5 c.c. trypsin, 20 c.c. 13 % alcohol,	20 c.c. 1 % Na_2CO_3	14.0
	(b)	5 c.c. trypsin, 20 c.c. water,	20 c.c. 1 % Na_2CO_3	25.2
		1.3 g. fibrin. Digestion 3 hours.				
	(a)	5 c.c. trypsin, 20 c.c. 13 % alcohol,	20 c.c. caseinogen	34.5
	(b)	5 c.c. trypsin, 20 c.c. water,	20 c.c. caseinogen	34.6
		Digestion 1.25 hours.				
11.	(a)	5 c.c. trypsin, 20 c.c. 14 % alcohol,	20 c.c. 1 % Na_2CO_3	13.7
	(b)	5 c.c. trypsin, 20 c.c. water,	20 c.c. 1 % Na_2CO_3	26.8
		1.2 g. fibrin. Digestion 2.5 hours.				
	(a)	5 c.c. trypsin, 20 c.c. 14 % alcohol,	20 c.c. caseinogen	32.5
	(b)	5 c.c. trypsin, 20 c.c. water,	20 c.c. caseinogen	32.5
		Digestion 1 hour.				
12.	(a)	5 c.c. trypsin, 20 c.c. 14 % alcohol,	20 c.c. 1 % Na_2CO_3	10.8
	(b)	5 c.c. trypsin, 20 c.c. water,	20 c.c. 1 % Na_2CO_3	24.6
		1 g. fibrin. Digestion 2 hours.				
	(a)	5 c.c. trypsin, 20 c.c. 14 % alcohol,	20 c.c. caseinogen	30.0
	(b)	5 c.c. trypsin, 20 c.c. water,	20 c.c. caseinogen	30.1
		Digestion 1 hour.				

These experiments are sufficient to show that alcohol, when present in percentages varying from 3 to 7, has a very marked inhibitory effect on the digestion of fibrin by trypsin but no such effect on the digestion of caseinogen. The amount of fibrin digested under these conditions varied from about 25 to 50 % of the amount digested in absence of alcohol, the proportion varying somewhat with different trypsin solutions and with varying percentages of alcohol. In no case was there any appreciable difference in the amount of caseinogen digested, beyond the limits of experimental error.

With higher percentages of alcohol the digestion of fibrin was in some cases entirely stopped, a fair amount of caseinogen still being digested, however.

						Digestion in c.c. of N/10 nitrogen
13.	(a)	1 c.c. trypsin, 20 c.c. 25 % alcohol,	20 c.c. 1 % Na_2CO_3	3.1
	(b)	1 c.c. trypsin, 20 c.c. water,	20 c.c. 1 % Na_2CO_3	27.2
		1 g. fibrin. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 25 % alcohol,	20 c.c. caseinogen	20.0
	(b)	1 c.c. trypsin, 20 c.c. water,	20 c.c. caseinogen	23.7
		Digestion 1 hour.				

						Digestion in cc. of N/10 nitrogen
14.	(a)	1 c.c. trypsin, 20 c.c. 25 % alcohol, 20 c.c. 1 % Na_2CO_3	4.8
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	25.2
		0.8 g. fibrin. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 25 % alcohol, 20 c.c. caseinogen	32.8
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	37.9
		Digestion 1.75 hours.				
15.	(a)	1 c.c. trypsin, 20 c.c. 50 % alcohol, 20 c.c. 1 % Na_2CO_3	0.0
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	23.6
		1 g. fibrin. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 50 % alcohol, 20 c.c. caseinogen	5.3
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	25.2
		Digestion 1 hour.				
16.	(a)	1 c.c. trypsin, 20 c.c. 50 % alcohol, 20 c.c. 1 % Na_2CO_3	0.0
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. 1 % Na_2CO_3	23.4
		1.2 g. fibrin. Digestion 3 hours.				
	(a)	1 c.c. trypsin, 20 c.c. 50 % alcohol, 20 c.c. caseinogen	4.6
	(b)	1 c.c. trypsin, 20 c.c. water, 20 c.c. caseinogen	28.2
		Digestion 1 hour.				

These experiments show that in presence of 25 % of alcohol the digestion of fibrin by trypsin is entirely inhibited, while digestion of caseinogen still proceeds to a limited extent. In presence of 12 % alcohol the amount of fibrin digested is from 10 to 20 % of the control, while the caseinogen digested amounts to about 85 % of the control.

Trypsin is well known to be very unstable under some circumstances, and it was considered possible that contact with dilute alcohol for some time might lead to an actual destruction of that part of the enzyme molecule which digests fibrin. The following experiments were carried out to test such a theory.

						Digestion in c.c. of N/10 nitrogen
17.	(a)	20 c.c. trypsin, 15 c.c. 15 % alcohol	} kept at 37° C. for 3 hours			
	(b)	20 c.c. trypsin, 15 c.c. water				
		2 c.c. of (a), 40 c.c. 0.5 % Na_2CO_3	31.2
		2 c.c. of (b), 40 c.c. 0.5 % Na_2CO_3	31.0
		1.3 g. fibrin. Digestion 3 hours.				
18.	(a)	20 c.c. trypsin, 5 c.c. 30 % alcohol	} kept at 37° C. for 3 hours			
	(b)	20 c.c. trypsin, 5 c.c. water				
		2 c.c. of (a), 40 c.c. 0.5 % Na_2CO_3	18.2
		2 c.c. of (b), 40 c.c. 0.5 % Na_2CO_3	18.3
		1.2 g. fibrin. Digestion 2 hours.				
19.	(a)	40 c.c. trypsin, 10 c.c. 30 % alcohol	} kept at 37° C. for 3 hours			
	(b)	40 c.c. trypsin, 10 c.c. water				
		1 c.c. of (a), 40 c.c. 0.5 % Na_2CO_3	14.0
		1 c.c. of (b), 40 c.c. 0.5 % Na_2CO_3	14.2
		1 g. fibrin. Digestion 3 hours.				
20.	(a)	15 c.c. trypsin, 10 c.c. 15 % alcohol	} kept at 37° C. for 3 hours			
	(b)	15 c.c. trypsin, 10 c.c. water				
		2 c.c. of (a), 40 c.c. 0.5 % Na_2CO_3	17.4
		2 c.c. of (b), 40 c.c. 0.5 % Na_2CO_3	17.0
		1 g. fibrin. Digestion 2.75 hours.				

						Digestion in c.c. of N/10 nitrogen
21.	(a)	15 c.c. trypsin, 10 c.c. 15 % alcohol	} kept at 37° C. for 3 hours			
	(b)	15 c.c. trypsin, 10 c.c. water				
		2 c.c. of (a), 40 c.c. 0.5 % Na_2CO_3		16.3
		2 c.c. of (b), 40 c.c. 0.5 % Na_2CO_3		16.2
		1 g. fibrin. Digestion 2.75 hours.				

No destruction whatever of the trypsin is caused by the action of 6 % alcohol, although the digestive action of the enzyme is reduced to 30 % or less of the normal amount by the presence of this proportion of alcohol.

A solid substrate such as fibrin might be rendered less digestible by prolonged treatment with concentrated alcohol, owing to the hardening thus brought about. Alcohol of under 30 %, however, could hardly be supposed to have such an effect, and a few experiments showed that after treatment with dilute alcohol fibrin was no less digestible by trypsin than previously.

						Digestion in c.c. of N/10 nitrogen
22.	(1)	Fibrin + 10 % alcohol	} kept at 37° C. for 3 hours			
	(2)	Fibrin + water				
		1 c.c. trypsin, 40 c.c. 0.5 % Na_2CO_3 , 1 g. fibrin (1)	19.3
		1 c.c. trypsin, 40 c.c. 0.5 % Na_2CO_3 , 1 g. fibrin (2)	18.1
		Digestion 2.5 hours.				
23.	(1)	Fibrin + 10 % alcohol	} kept at 37° C. for 19 hours			
	(2)	Fibrin + 10 % alcohol				
		1 c.c. trypsin, 40 c.c. 0.5 % Na_2CO_3 , 1 g. fibrin (1)	22.9
		1 c.c. trypsin, 40 c.c. 0.5 % Na_2CO_3 , 1 g. fibrin (2)	20.2
		Digestion 3 hours.				

The fibrin which was to be treated with alcohol in these experiments was first washed with alcohol in order to remove any adherent moisture. It will be seen that after treatment with 10 % alcohol fibrin is apparently slightly more readily attacked by trypsin than previously.

The action of trypsin on fibrin and on caseinogen is affected by dilute alcohol to such different degrees that it is reasonable to suppose either that there are two enzymes concerned in the digestion of these proteins or that different groups of the same enzyme molecule take part in the hydrolysis of the different proteins. In the latter case the groups which digest fibrin are very much more easily inhibited by alcohol than the groups which digest caseinogen.

The theory that different side chains in the molecule of an enzyme are responsible for different functions is used to explain the zymoid modification of enzymes. Some observers also, for example, Nencki and Sieber [1901], hold that the behaviour of pepsin and rennin under varying conditions can best be explained on the theory that only one enzyme is concerned here, with different side chains responsible for the proteolytic and milk coagulating functions. Vernon [1903, 1] also considers this probable in the case of the milk coagulating and proteolytic actions of trypsin.

Hitherto it has apparently been assumed that one enzyme "trypsin" is responsible for the digestion of fibrin and caseinogen by pancreatic extracts.

In this case the function is the same (hydrolysis of a protein to form simpler products), but it would seem that different side chains may be necessary for the hydrolysis of different proteins.

SUMMARY.

Alcohol when present to the extent of 3 % and upwards markedly inhibits the action of trypsin on fibrin. The digestion of caseinogen by trypsin is not affected until the concentration reaches 10 %. The action of alcohol is not due to the destruction of the trypsin, since on suitable dilution of the mixture of trypsin and alcohol the digestion of fibrin is as great as in the control.

Fibrin is not rendered less digestible by contact with dilute alcohol, but seems to be slightly more readily dissolved by trypsin than previously.

If "trypsin" is a single enzyme the digestion of fibrin and caseinogen is probably carried on by different side chains, those digesting fibrin being much more readily affected by alcohol than the others.

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XXIV. STUDIES ON COAGULATION. I. ON THE VELOCITY OF GELATION AND HYDROLYSIS OF GELATIN SOL.

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I. THE GELATION VELOCITY OF GELATIN SOL.

GELATION of hydrophilic colloids has frequently been studied in regard to its 'gelation time' (usually called the coagulation time) *i.e.* the time necessary for the gelatinising of a sol. The definition of 'gelation time,' however, is somewhat arbitrary and differs with the observer. Some observers consider a sol is gelatinised, when the vessel containing it can be inverted without spilling. Others consider it gelatinised, when it adheres as an amorphous mass to a rod inserted in it. Still others consider it gelatinised, when a small glass bead thrown into it stops half-way down. Gelation, however, is not a sudden change of state, but a kinetic process with respect to time. Therefore it is more important and fundamental to study the course of a whole process of gelation than to study simply the gelation time.

It is not yet known exactly, what change of state the colloidal constituents undergo in the process of gelation. The process is called gelation only when a hydrophilic sol, separating into two phases, gradually increases its viscosity until it becomes an amorphous solid mass throughout. Thus the degree of gelation can be estimated by measuring the increment of viscosity, and the rate at which the viscosity increases is called the 'gelation velocity.'

Von Schröder [1903] studied this problem as follows. He heated a gelatin sol at 100° for a certain time, and then cooled it suddenly to 25°. He now kept the sol at this latter temperature, and measured its viscosity twice, first, after a lapse of five minutes, and again, after sixty minutes. The second value was of course larger than the first one. He called the difference of these two viscosity values $\Delta\eta$, and the time interval between the measurements (55 minutes) Δt , and assumed that $\frac{\Delta\eta}{\Delta t}$ represented the coagulability of the sol. He found that the longer the sol was kept at 100°, the smaller were both the viscosity at the first measurement and $\frac{\Delta\eta}{\Delta t}$.

Levites [1908] later studied this problem in the same way. He measured the viscosity of a sol several times in the course of its gelation, and found that the gelation velocity, $\frac{d\eta}{dt}$, was a constant independent of the time, so that the viscosity is a linear function of the time. He studied the process, however, only during the first 1.5 hours of a gelation, which is a relatively brief period when it is remembered that the whole process, according to his own statement, occupied from 11 to 24 hours. His conclusion, therefore, is necessarily somewhat insufficiently supported by experiment.

The present writer took the same problem. The method and procedure followed by him were as follows. A weighed amount of dried commercial gelatin is put into water in such proportions as to have a desired percentage solution when dissolved. This mixture is kept in a cool place in a flask for a certain time in order to allow the solid gelatin to swell sufficiently. It is then placed in a thermostat at 70° or 80°, and by thorough shaking is brought to a homogeneous sol in a few minutes. The flask is fitted with a condenser to prevent the sol from evaporating. At successive intervals after the beginning of heating 5 cc. of the sol are taken out of the flask, and put into an Ostwald viscosimeter kept in another thermostat at 18° and allowed to gelatinise at this temperature. Its viscosity coefficient is then determined frequently over a considerable period of time. The first determination is made at the end of the first six minutes, preliminary experiments showing that the temperature of the sol brought into the 18° thermostat falls to this temperature in five minutes. The initial viscosity coefficient, *i.e.* the viscosity coefficient at the initial time of gelation is found by extrapolation, assuming that the process of gelation begins regularly as soon as the sol is brought into the 18° thermostat.

Since the successive portions are taken out of one and the same sol, they differ only in the length of time they have been previously heated, and the whole course of gelation of each of them is studied in detail, since it is desired to study the influence of previous heating on the process of gelation, as well as the process of gelation itself. The observations give the relative viscosity coefficient, that of water at 18° being taken as unity. The absolute viscosity coefficient can then be easily calculated if desired.

The results of experiments with a 3 per cent. gelatin sol are shown in Fig. 1. The quantities $\eta - \eta_i$ are taken as ordinates and the time t as abscissa, η and η_i being the viscosity coefficients at the time t and at the initial time of gelation respectively. Curves 1, 2, 3, 4, 5 and 6 are viscosity-time curves for portions previously heated for six minutes, 1, 3, 6, 11 and 20 hours respectively at 80°. Fig. 2 is another example of a 3 per cent. sol which was heated at 70° instead of 80°, curves 1, 2, 3, 4, 5, 6 and 7 corresponding to portions heated for 6 minutes, 2, 6, 16, 20, 30 and 39 hours respectively.

It is seen clearly from these figures that the longer the sol is previously heated, the smaller is the initial gelation velocity, a result in agreement with

that found by von Schröder. This disagrees, however, with Levites since the viscosity fails to be a linear function of the time in all cases. When the sol is heated for a relatively short time, the gelation velocity, $\frac{d\eta}{dt}$, increases with the time, so that the curve is convex towards the t -axis (Fig. 1, Nos. 1 and 2). For the sake of convenience let us call this the first case. On the contrary,

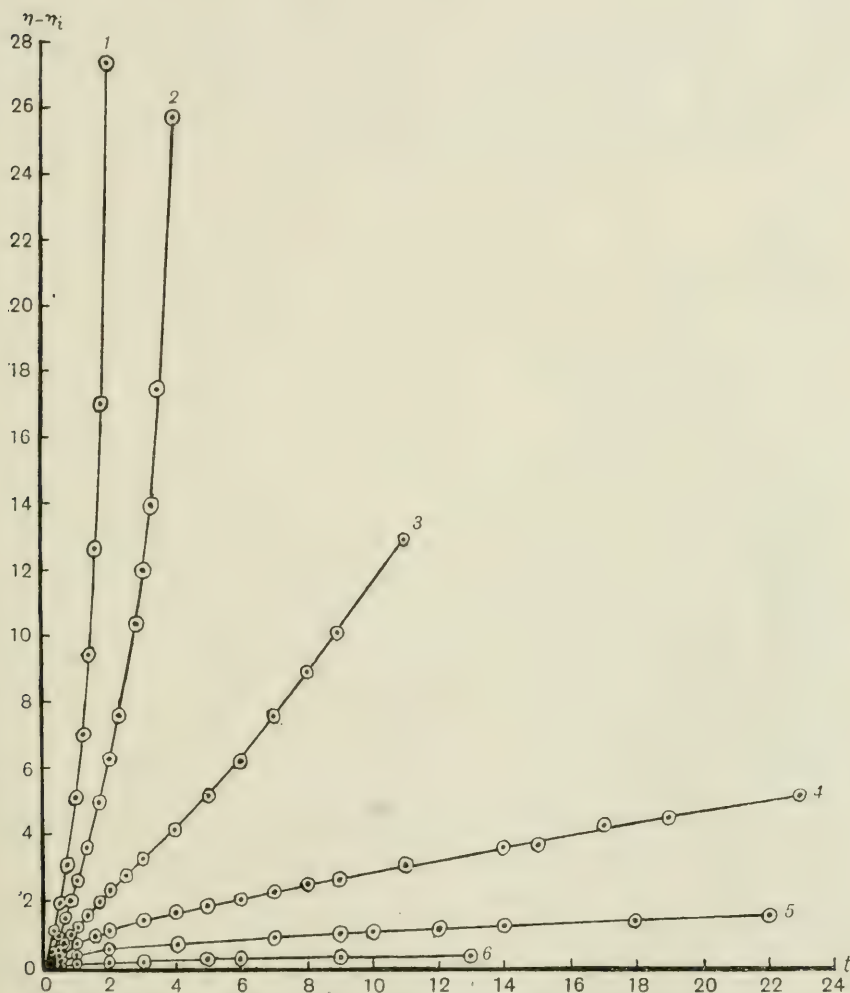


Fig. 1. Gelation of 3 per cent. gelatin sol. (previously heated at 80°).

when the sol is heated relatively longer, the gelation velocity decreases with the time, so that the curve is concave towards the t -axis (Fig. 1, Nos. 4, 5 and 6), which we may call the second case. At some intermediate 'critical' stage the gelation velocity is constant, so that the curve is a straight line. Fig. 1, No. 3 approximates to this case.

It is generally believed that α -glutin, the actual gelatinising constituent of gelatin, is gradually hydrolysed, by heating, to β -glutin which has no tendency to gelatinise even at low temperatures, and as a matter of fact, the longer the sol is heated, the further the hydrolysis goes. Adopting this point of view, therefore, we may say that the higher the concentration of α -glutin, the larger the initial gelation velocity; that when its concentration is higher than a certain critical value, the gelation velocity increases with the time until the viscosity grows beyond bounds; and that when the concentration is lower than this critical value, the gelation velocity decreases with the time, until the viscosity ultimately reaches a finite, limiting value. It is not clear whether the concentration of β -glutin affects these facts or not.

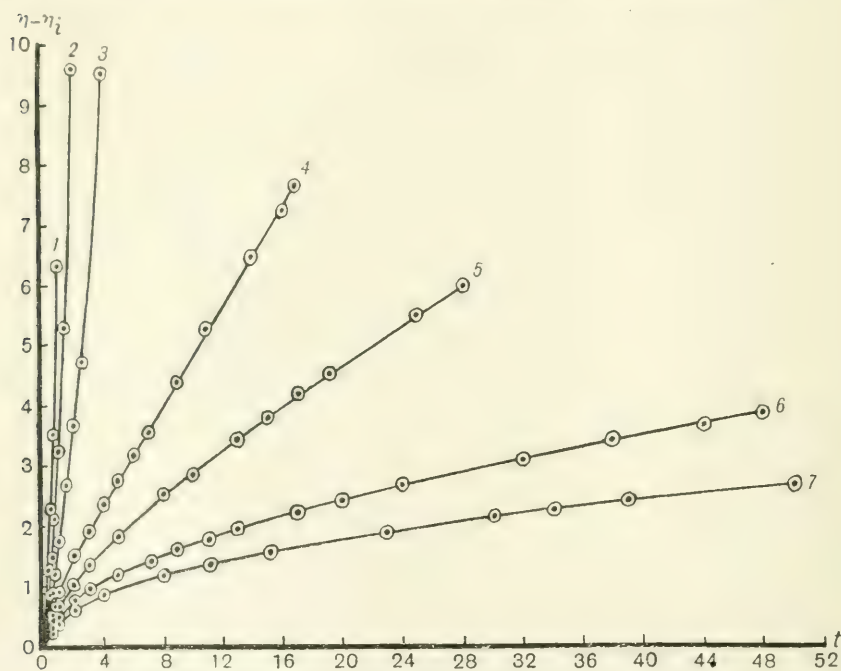


Fig. 2. Gelation of 3 per cent. gelatin sol. (previously heated at 70°).

These conclusions are supported by similar experiments with a 1 per cent. gelatin sol represented in Fig. 3. Curves 1, 2, 3 and 4 are viscosity-time curves for portions previously heated at 80° for 0.1, 1.2, 4 and 11 hours respectively. We see that the gelation velocity decreases with the time even when the sol has been heated for only a short period. That is to say, the gelation belongs to the second, and never to the first case. Obviously the concentration of α -glutin in this case has been lower than the critical concentration from the beginning.

The numerical value of the critical concentration of α -glutin cannot be found, since we do not know the concentration of α -glutin in solid gelatin.

The curves of Figs. 1, 2 and 3 suggested the empirical equation

$$\eta - \eta_i = \frac{\mu t}{1 + \frac{\mu}{\lambda} t} \dots\dots\dots(1)$$

both μ and λ being constants. To test this equation we may write it as

$$\frac{1}{\eta - \eta_i} = \frac{1}{\mu} \frac{1}{t} + \frac{1}{\lambda} \dots\dots\dots(2)$$

a linear equation in $\frac{1}{\eta - \eta_i}$ and $\frac{1}{t}$. It is then found that equation (2) is a good fit. The straight line (the figure is not given here) for the critical case $\lambda = \infty$ passes through the origin in each case, the straight lines below it ($\lambda < 0$) corresponding to samples heated for a shorter period, and the straight lines above it ($\lambda > 0$) corresponding to samples heated for a longer period.

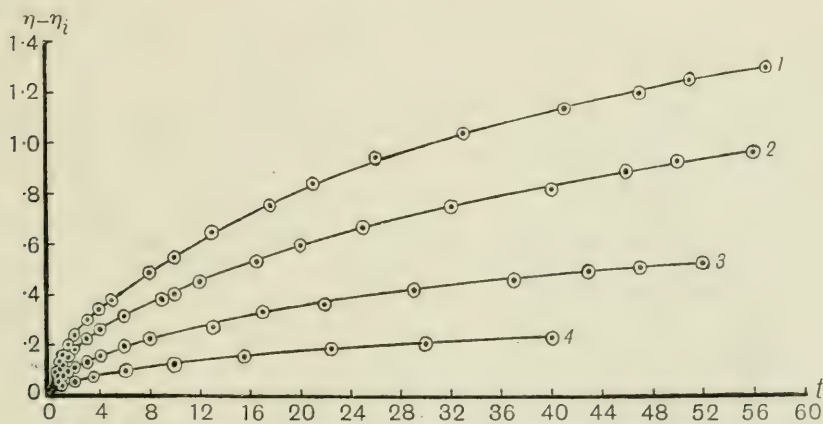


Fig. 3. Gelation of 1 per cent. gelatin sol. (previously heated at 80°).

The constant μ is plainly the value of $\frac{d\eta}{dt}$ at $t = 0$, *i.e.* the initial gelation velocity; we may regard it as increasing with the concentration of α -glutin. It ranges from 0 to ∞ . As to the constant λ we may regard it as increasing with the concentration of α -glutin up to the critical concentration where it becomes infinite. For slightly higher concentrations λ is large and negative and decreases through negative values to a definite limit. This is more or less equivalent to regarding $\frac{1}{\lambda}$ as given by $\frac{1}{c} - \frac{1}{C}$, where c and C are respectively the concentration of α -glutin and the critical concentration. In terms of the curves we may say that λ , when positive, is the value of $\eta - \eta_i$ attained after a long interval of time, while, when λ is negative, the $\eta - \eta_i$ increases beyond bounds after a time given by $t = -\frac{\lambda}{\mu}$. In this latter case it is reasonable to speak of a 'gelation time' but not in the former case. As we see from equation (2) the constants $\frac{1}{\mu}$ and $\frac{1}{\lambda}$ may be taken directly from the straight lines of its graph; $\frac{1}{\mu}$ represents the tangent slope and $\frac{1}{\lambda}$ the intercept on the vertical axis.

Equation (1) may be looked upon as the solution of the differential equation

$$\frac{d}{dt}[\lambda - (\eta - \eta_i)] = \frac{\mu}{\lambda^2} [\lambda - (\eta - \eta_i)]^2 \dots \dots \dots (3).$$

Now it may be shown that not only the equation (2), but also the solutions of the equations of more general type

$$-\frac{d}{dt}[\lambda - (\eta - \eta_i)] = \frac{\mu}{\lambda^n} [\lambda - (\eta - \eta_i)]^n,$$

where n is an integer, represent curves of the same general character as those given by the above experiments; the significance of μ and λ in these new equations being the same as before. Two solutions of this differential equation of general type are

$$(a) \quad \eta - \eta_i = \lambda (1 - e^{-t}), \quad \text{when } n = 1,$$

$$\text{and, } (b) \quad \frac{1}{[\lambda - (\eta - \eta_i)]^2} = \frac{2\mu}{\lambda^3} t + \frac{1}{\lambda^2}, \quad \text{when } n = 3.$$

Tests were made with these solutions and it was found that they accorded with the observations only for the first shorter period of gelation, and therefore they may reasonably be rejected in favour of equation (1).

Comparison of the values observed and calculated by equation (1) is to be seen in Tables 1, 2 and 3 which correspond to Figs. 1, 2 and 3 respectively.

It is seen from these tables that equation (1) holds for the first four or five hours of gelation, but that at later periods, the viscosity observed increases more rapidly than is given by this equation. Moreover, as can be seen in Fig. 1, curve 3, a gelation starting as a second case, but very near the critical case, may change gradually into the first case, after a certain lapse of time, so that the curve has an inflexion point. Therefore, the statements given above, although holding exactly for earlier periods of gelation, do not hold for later periods. An equation which holds for the whole course of gelation has not yet been found.

The equation (3) is an equation of the second order reaction with respect to the term $\lambda - (\eta - \eta_i)$. If it is assumed that this term is directly proportional to the concentration of α -glutin, the nature of gelation can be discussed as follows.

From the kinetic theory point of view, the particles of a sol are continuously colliding with each other, and gelation can be regarded as being due to the fact that the particles, or at least a certain proportion of them, unite to form an aggregate particle on coming into contact with each other. In the earlier periods of gelation such a union would occur mainly between two individual particles, the reaction thus being of the second order, as given by equation (3). Later, however, when the number of such aggregate particles becomes larger, the probability of union between an aggregate particle and a single particle, as well as between two aggregate particles, increases, so that the viscosity increases more rapidly than indicated by equation (1). These aggregate particles again unite with others, and become larger and larger

Table 1. *Gelation of 3 per cent. gelatin sol at 18° (Fig. 1).
(Previously heated at 80°.)*

$\eta - \eta_i$				$\eta - \eta_i$				
t (hours)	η	obs.	calc.	t (hours)	η	obs.	calc.	
$\begin{cases} \mu = 3.2 \\ \lambda = -8.6 \end{cases}$				$\begin{cases} \mu = 0.80 \\ \lambda = 2.7 \end{cases}$				
No. 1 (Curve 1) Heated for 6 minutes	0	2.15	0	0	0	1.75	0	
	0.1	2.47	0.32	0.33	0.1	1.83	0.08	0.08
	0.3	3.22	1.07	1.08	0.2	1.90	0.15	0.15
	0.5	4.10	1.95	1.96	0.5	2.11	0.36	0.35
	0.7	5.20	3.05	3.03	1.0	2.37	0.62	0.62
	1.0	7.28	5.13	5.10	1.6	2.62	0.87	0.87
	1.2	9.10	6.95	6.95	2.0	2.74	0.99	1.00
	1.4	11.52	9.37	9.37	3.0	3.02	1.27	1.27
	1.6	14.75	12.6	12.6	4.0	3.32	1.57	1.47
	1.8	19.05	16.9	17.5	5.0	3.50	1.75	1.61
2.05	29.5	27.4	27.7	6.0	3.77	2.02		
$\begin{cases} \mu = 2.2 \\ \lambda = -14.7 \end{cases}$				7.0	3.98	2.23		
No. 2 (Curve 2) Heated for 1 hour	0	2.01	0	0	8.0	4.23	2.48	
	0.1	2.23	0.22	0.22	9.0	4.35	2.60	
	0.2	2.48	0.47	0.45	11.0	4.84	3.09	
	0.4	2.96	0.95	0.94	14.0	5.28	3.53	
	0.6	3.48	1.47	1.45	15.0	5.48	3.73	
	0.8	4.02	2.01	2.00	17.0	5.90	4.15	
	1.0	4.58	2.57	2.59	19.0	6.20	4.45	
	1.3	5.57	3.56	3.55	23.0	6.88	5.13	
	1.7	7.05	5.04	5.02	$\begin{cases} \mu = 0.45 \\ \lambda = 0.90 \end{cases}$			
	2.0	8.25	6.24	6.27	0	1.66	0	0
	2.3	9.61	7.60	7.71	0.1	1.70	0.04	0.04
	2.8	12.3	10.3	10.4	0.3	1.78	0.12	0.12
	3.0	14.0	12.0	12.0	0.5	1.84	0.18	0.18
	3.3	15.9	13.9	14.3	1.0	1.96	0.30	0.30
	3.55	19.3	17.3	16.6	2.0	2.11	0.45	0.45
4.0	27.7	25.7	21.9	4.0	2.26	0.60	0.60	
$\begin{cases} \mu = 1.27 \\ \lambda = 20.0 \end{cases}$				7.0	2.48	0.82	0.70	
No. 3 (Curve 3) Heated for 3 hours	0	1.87	0	0	9.0	2.56	0.90	
	0.2	2.12	0.25	0.25	10.0	2.61	0.95	
	0.4	2.40	0.53	0.50	12.0	2.73	1.07	
	0.6	2.60	0.73	0.73	14.0	2.83	1.17	
	0.8	2.84	0.97	0.97	18.0	2.95	1.29	
	1.0	3.07	1.20	1.19	22.0	3.04	1.38	
	1.3	3.43	1.56	1.53	$\begin{cases} \mu = 0.16 \\ \lambda = 0.46 \end{cases}$			
	1.7	3.83	1.96	1.95	0	1.57	0	0
	2.0	4.12	2.25	2.25	0.2	1.60	0.03	0.03
	2.5	4.60	2.73	2.74	0.5	1.64	0.07	0.07
	3.0	5.06	3.19	3.20	1.0	1.69	0.12	0.12
	4.0	5.98	4.11	4.05	2.0	1.76	0.19	0.19
	5.0	6.96	5.09	4.82	3.0	1.80	0.23	0.23
	6.0	8.09	6.22	5.51	5.0	1.86	0.29	0.29
	7.0	9.45	7.58		6.0	1.89	0.32	0.31
8.0	10.77	8.90		9.0	1.94	0.37	0.35	
9.0	11.96	10.09		13.0	1.98	0.41		
11.0	14.75	12.88						

Table 2. Gelation of 3 per cent. gelatin sol at 18° (Fig. 2).
(Previously heated at 70°.)

		t		$\eta \cdot \eta_i$	
		(hours)	η	obs.	calc.
				$\mu = 3.52$ $\lambda = -7.9$	
No. 1 (Curve 1)	Heated for 6 minutes	0	2.00	0	0
		0.1	2.36	0.36	0.37
		0.3	3.23	1.23	1.22
		0.5	4.27	2.27	2.26
		0.7	5.55	3.55	3.58
1.0		8.35	6.35	6.35	
				$\mu = 2.50$ $\lambda = -10.4$	
No. 2 (Curve 2)	Heated for 2 hours	0	1.93	0	0
		0.1	2.18	0.25	0.26
		0.3	2.76	0.83	0.81
		0.5	3.35	1.42	1.42
		0.7	4.03	2.10	2.10
1.0		5.18	3.25	3.29	
1.4		7.20	5.27	5.27	
2.0		11.53	9.60	9.61	
				$\mu = 1.63$ $\lambda = 30.0$	
No. 3 (Curve 3)	Heated for 6 hours	0	1.84	0	0
		0.1	2.01	0.17	0.16
		0.3	2.33	0.49	0.50
		0.5	2.69	0.85	0.84
		0.7	3.01	1.17	1.19
1.0		3.56	1.72	1.72	
1.5		4.50	2.66	2.66	
2.0		5.49	3.65	3.66	
2.5		6.58	4.74	4.72	
4.0		11.37	9.53	8.34	
				$\mu = 1.10$ $\lambda = 4.5$	
No. 4 (Curve 4)	Heated for 16 hours	0	1.72	0	0
		0.1	1.83	0.11	0.11
		0.3	2.04	0.32	0.31
		0.5	2.21	0.49	0.49
		0.7	2.37	0.65	0.66
1.0		2.60	0.88	0.88	
2.0		3.20	1.48	1.48	
3.0		3.62	1.90	1.90	
4.0		4.08	2.36	2.22	
5.0		4.48	2.76		
6.0		4.90	3.18		
7.0		5.28	3.56		
9.0		6.08	4.36		
11.0		6.93	5.21		
14.0		8.19	6.47		
16.0		8.98	7.26		
17.0		9.37	7.65		
				$\mu = 1.30$ $\lambda = 0$	
No. 5 (Curve 5)	Heated for 20 hours	0	1.70	0	0
		0.1	1.80	0.10	0.095
		0.3	1.96	0.26	0.26
		0.5	2.10	0.40	0.40
		1.0	2.36	0.66	0.66
2.0		2.69	0.99	0.99	
3.0		3.03	1.33	1.18	
5.0		3.53	1.83		
8.0		4.19	2.49		
10.0		4.55	2.85		
13.0		5.11	3.41		
15.0		5.48	3.78		
17.0		5.85	4.15		
19.0		6.21	4.51		
25.0		7.20	5.50		
28.0		7.64	5.94		
				$\mu = 0.85$ $\lambda = 1.30$	
No. 6 (Curve 6)	Heated for 30 hours	0	1.63	0	0
		0.1	1.71	0.08	0.08
		0.3	1.85	0.22	0.21
		0.5	1.95	0.32	0.32
		1.0	2.14	0.51	0.51
2.0		2.37	0.74	0.74	
3.0		2.53	0.90	0.86	
5.0		2.79	1.16		
7.0		2.99	1.36		
9.0		3.21	1.58		
11.0		3.39	1.76		
13.0		3.56	1.93		
17.0		3.83	2.20		
20.0		4.04	2.41		
24.0		4.27	2.64		
32.0		4.72	3.09		
38.0		5.04	3.41		
44.0		5.30	3.67		
48.0		5.50	3.87		
				$\mu = 0.83$ $\lambda = 0.97$	
No. 7 (Curve 7)	Heated for 39 hours	0	1.61	0	0
		0.1	1.69	0.08	0.08
		0.3	1.81	0.20	0.20
		0.5	1.90	0.29	0.29
		1.0	2.05	0.44	0.45
2.0		2.22	0.61	0.61	
4.0		2.43	0.82	0.75	
8.0		2.76	1.15		
11.0		2.95	1.34		
15.0		3.16	1.55		
23.0		3.47	1.86		
30.0		3.74	2.13		
34.0		3.87	2.26		
39.0		4.02	2.41		
50.0		4.26	2.65		

Table 3. *Gelation of 1 per cent. gelatin sol at 18° (Fig. 3).*
(Previously heated at 80°.)

$\eta - \eta_i$				$\eta - \eta_i$					
t	η	obs.	calc.	t	η	obs.	calc.		
(hours)				(hours)					
$\begin{cases} \mu = 0.21 \\ \lambda = 0.57 \end{cases}$				$\begin{cases} \mu = 0.10 \\ \lambda = 0.23 \end{cases}$					
No. 1 (Curve 1) Heated for 6 minutes	0	1.26	0	0	0	1.22	0	0	
	0.1	1.28	0.02	0.02	0.1	1.23	0.01	0.01	
	0.2	1.30	0.04	0.04	0.3	1.25	0.03	0.03	
	0.5	1.35	0.09	0.09	0.6	1.27	0.05	0.05	
	0.8	1.39	0.13	0.13	1.0	1.29	0.07	0.07	
	1.0	1.42	0.16	0.15	1.5	1.31	0.09	0.09	
	1.5	1.46	0.20	0.20	2.0	1.33	0.11	0.11	
	2.0	1.50	0.24	0.24	No. 3	3.0	1.35	0.13	0.13
	3.0	1.56	0.30	0.30	(Curve 3)	4.0	1.37	0.15	0.15
	4.0	1.60	0.34	0.34	Heated	6.0	1.41	0.19	0.17
	5.0	1.64	0.38	0.37	for	8.0	1.44	0.22	0.18
	8.0	1.75	0.49	0.43	4 hours	13.0	1.49	0.27	
	10.0	1.81	0.55			17.0	1.55	0.33	
	13.0	1.90	0.64			22.0	1.58	0.36	
	17.5	2.01	0.75			29.0	1.63	0.41	
	21.0	2.10	0.84			37.0	1.67	0.45	
	26.0	2.20	0.94			43.0	1.71	0.49	
	33.0	2.29	1.03			47.0	1.72	0.50	
	41.0	2.39	1.13			52.0	1.74	0.52	
47.0	2.45	1.19							
51.0	2.50	1.24						$\begin{cases} \mu = 0.037 \\ \lambda = 0.15 \end{cases}$	
57.0	2.55	1.29							
$\begin{cases} \mu = 0.17 \\ \lambda = 0.40 \end{cases}$				$\begin{cases} \mu = 0.037 \\ \lambda = 0.15 \end{cases}$					
No. 2 (Curve 2) Heated for 1.2 hours	0	1.244	0	0	0	1.20	0	0	
	0.1	1.26	0.016	0.016	0.3	1.21	0.01	0.01	
	0.3	1.29	0.046	0.045	0.6	1.22	0.02	0.02	
	0.6	1.325	0.08	0.08	1.0	1.23	0.03	0.03	
	0.8	1.34	0.10	0.10	2.0	1.25	0.05	0.05	
	1.0	1.36	0.12	0.12	3.5	1.27	0.07	0.07	
	1.5	1.39	0.15	0.15	6.0	1.29	0.09	0.09	
	2.0	1.42	0.18	0.18	10.0	1.32	0.12	0.11	
	3.0	1.46	0.22	0.22	15.5	1.35	0.15	0.12	
	4.0	1.50	0.26	0.25	22.5	1.38	0.18		
	6.0	1.55	0.31	0.29	30.0	1.40	0.20		
	9.0	1.62	0.38	0.32	40.0	1.42	0.22		
	10.0	1.64	0.40						
	12.0	1.69	0.45						
	16.5	1.77	0.53						
	20.0	1.83	0.59						
	25.0	1.90	0.66						
	32.0	1.98	0.74						
	40.0	2.05	0.81						
46.0	2.12	0.88							
50.0	2.16	0.92							
56.0	2.20	0.96							

with the lapse of time. They contain, of course, a certain amount of water, gelatin being a hydrophil colloid.

This hypothesis seems very probable, its only weakness being the assumption stated above. It can, indeed, be reasonably regarded that the term $\lambda - (\eta - \eta_i)$ is an increasing function of the concentration of α -glutin. But there is no theoretical or experimental basis for the assumption that the former is directly proportional to the latter.

Therefore the only conclusion which can be definitely deduced from the results of these experiments is that the gelation is, in its early stage at any rate, a second order reaction with respect to the term $\lambda - (\eta - \eta_i)$.

II. ON THE HYDROLYSIS OF GELATIN BY HEATING.

Gelatin sol undergoes an irreversible change when it is heated at a high temperature. This change is believed to be a hydrolysis of α -glutin, producing β -glutin, as already mentioned in the foregoing section. Commercial gelatin contains to begin with a certain amount of β -glutin, for heat is applied in its production. Moreover, when it is brought to a sol, heat is again applied, so that a part of the α -glutin changes to β -glutin. Hence the concentration of α -glutin in a gelatin sol of known percentage is quite unknown and varies with the sample used and the conditions of its preparation.

The dynamic study of the hydrolysis of gelatin is merely a study of the variation of the concentration of the α -glutin or its hydrolysis products with the time. However, we have as yet no method of determining the concentration of α -glutin or of β -glutin separately. Moreover, β -glutin may reasonably be regarded, not as a definite substance, but as a mixture of the products at various stages of the hydrolysis caused by heating.

The determination of the viscosity of the sol might be regarded as a method for studying its hydrolysis. But, even when β -glutin is assumed to be a single substance, a gelatin sol is still a mixture of two colloids, namely α -glutin and β -glutin. Now the viscosity of a mixture is not yet known as a definite function of the viscosities and concentrations of its constituents; at least it is not a linear function of the concentrations of the constituents, except in the case of dilute solutions. Therefore the hydrolysis of gelatin cannot be determined by measuring changes in its viscosity. In fact we do not know as yet of any measurable properties of a gelatin sol whose rate of change is proportional to the rate of change of the concentration of α -glutin in the process of its hydrolysis caused by heating.

Now, as a by-product of the experiments of the foregoing chapter, a study was made of the change of viscosity of the sol during the course of its hydrolysis. This study was made, however, for its own intrinsic interest, and not for its bearing on the process of the hydrolysis.

The initial viscosities η_i of the previous chapter are merely the viscosities of a mixture of α -glutin and β -glutin at successive periods of its hydrolysis, measured, however, at a lower temperature, viz. 18°. We shall, in what

follows, represent the time of heating by τ , to distinguish it from the time t in the course of subsequent gelation.

Von Schröder [1903] stated that the change in the viscosity of the hydrolysing gelatin sol, which he measured at 25°, was represented by an equation of the first order reaction, viz.:

$$-\frac{d\eta_i}{d\tau} = k_1 (\eta_i - \eta_{i\infty}),$$

i.e.

$$k_1 = \frac{1}{\tau} \ln \frac{\eta_{i0} - \eta_{i\infty}}{\eta_i - \eta_{i\infty}},$$

where η_{i0} and $\eta_{i\infty}$ are respectively the values of η_i at the beginning of the heating and after a long period of heating, while k_1 is a constant usually called the velocity coefficient.

Levites [1908], on the other hand, stated that this equation did not hold for his experiments with gelatin, agar and the sodium salt of α -thymonucleic acid. This equation fails, too, to represent correctly the results of the present experiments, whereas the equation of the third order reaction

$$-\frac{d\eta_i}{d\tau} = k_3 (\eta_i - \eta_{i\infty})^3$$

i.e.

$$k_3 = \frac{1}{2\tau} \left[\frac{1}{(\eta_i - \eta_{i\infty})^2} - \frac{1}{(\eta_{i0} - \eta_{i\infty})^2} \right]$$

or

$$k_3 = \frac{1}{2(\tau_1 - \tau_2)} \left[\frac{1}{(\eta_{i1} - \eta_{i\infty})^2} - \frac{1}{(\eta_{i2} - \eta_{i\infty})^2} \right] \left. \vphantom{\begin{matrix} k_3 = \frac{1}{2\tau} \left[\frac{1}{(\eta_i - \eta_{i\infty})^2} - \frac{1}{(\eta_{i0} - \eta_{i\infty})^2} \right] \\ k_3 = \frac{1}{2(\tau_1 - \tau_2)} \left[\frac{1}{(\eta_{i1} - \eta_{i\infty})^2} - \frac{1}{(\eta_{i2} - \eta_{i\infty})^2} \right] \end{matrix}} \right\} \dots\dots\dots(4)$$

where k_3 is a velocity coefficient varying with the temperature of heating and the concentration of α -glutin is found to be satisfactory. This can be seen from Tables 4, 5 and 6, in which the η_i (calc.) column is calculated from equation (4) using a mean value of k_3 , and k_2 is the velocity coefficient corresponding to the equation of the second order reaction, *i.e.*

$$k_2 = \frac{1}{\tau_1 - \tau_2} \left[\frac{1}{\eta_{i1} - \eta_{i\infty}} - \frac{1}{\eta_{i2} - \eta_{i\infty}} \right].$$

Table 4. *Hydrolysis of 3 per cent. gelatin sol heated at 80°.*
(Data of η_i taken from Table 1.)

τ (hours)	η_i	η_i (calc.)	k_3	k_2	$\cdot 434k_1$
0.1	2.15	—	—	—	—
1.0	2.01	2.02	0.33	0.26	0.088
3.0	1.87	1.86	0.29	0.20	0.060
6.0	1.75	1.75	0.30	0.18	0.047
11.0	1.66	1.65	0.29	0.15	0.034
20.0	1.57	1.57	0.31	0.13	0.025
∞	1.30	—	—	—	—
Mean			0.304		

Table 5. *Hydrolysis of 3 per cent. gelatin sol heated at 70°.*
(Data of η_i taken from Table 2.)

τ (hours)	η_i	η_i (calc.)	k_3	k_2	$\cdot 434k_1$
0.1	2.00	—	—	—	—
2.0	1.93	1.935	0.126	0.083	0.024
6.0	1.84	1.843	0.118	0.072	0.019
16.0	1.72	1.719	0.114	0.060	0.014
20.0	1.70	1.689	0.106	0.054	0.012
30.0	1.63	1.635	0.119	0.053	0.011
39.0	1.61	1.602	0.108	0.046	0.009
∞	1.30	—	—	—	—
Mean			0.115		

Table 6. *Hydrolysis of 1 per cent. gelatin sol heated at 80°.*
(Data of η_i taken from Table 3.)

τ (hours)	η_i	η_i (calc.)	k_1	k_2	$\cdot 434k_1$
0.1	1.26	—	—	—	—
1.2	1.244	1.244	10.4	1.16	0.057
4.0	1.22	1.221	11.1	1.07	0.045
11.0	1.20	1.198	9.6	0.76	0.028
∞	1.14	—	—	—	—
		Mean	10.4		

CONCLUSIONS.

1. The change of viscosity η of gelatin sol with respect to time t in the early stages of its gelation is represented by the equation

$$-\frac{d}{dt}[\lambda - (\eta - \eta_i)] = \frac{\mu}{\lambda^2} [\lambda - (\eta - \eta_i)]^2,$$

i.e.

$$\eta - \eta_i = \frac{\mu t}{1 + \frac{\mu}{\lambda} t},$$

μ being the rate of increase of viscosity at the initial moment of gelation, increasing with the initial concentration of the α -glutin. The constant λ is also larger, the larger the initial concentration of α -glutin, being positive or negative according as the initial concentration is below or above a certain critical concentration. When positive, λ is the asymptotic value of the viscosity attained after a long time. When λ is negative, gelation reaches its final stage after a finite time given by $t = -\frac{\lambda}{\mu}$.

The bearing of the above equation on the nature of gelation is discussed at some length.

2. The change of viscosity η_i of gelatin sol with respect to time τ in the course of its hydrolysis caused by heating is found to be represented by the equation,

$$-\frac{d\eta_i}{d\tau} = k(\eta_i - \eta_{i\infty})^3,$$

i.e.

$$2k\tau = \frac{1}{(\eta_i - \eta_{i\infty})^2} - \frac{1}{(\eta_{i0} - \eta_{i\infty})^2}.$$

The writer wishes to express his thanks to Dr Yoshio Ishida for some valuable suggestions which assisted him in finding the empirical gelation-formula adopted. Further studies on the process of gelation with respect to concentration and temperature will be reported in a following paper.

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XXV. NITROGEN PARTITION IN THE URINE OF THE RACES IN SINGAPORE.

By JAMES ARGYLL CAMPBELL.

(Received July 7th, 1919.)

INTRODUCTION.

THE observations here recorded form the continuation of a research directed mainly to the question of the nitrogen partition in the urine of individuals belonging to different races, including a Brahmin, a Chinese, a Tamil, a European, a Malay, a Hindoo, a Eurasian, a Bengali and a Sikh. The European has been a lecturer for more than five years and the other subjects have studied medicine for at least two years at the local school.

The first five of these subjects have been under observation since October 1916. Some of the results dealing with their diet, nutrition and excretion have been published [1917] and where comparable they support the evidence of the present work.

PREVIOUS RESEARCH.

A good deal of research has been published connected with diet and nutrition in various tropical climates but scarcely any dealing in detail with the nitrogen partition, the only searching work being that carried out by Young [1915], who studied the protein metabolism of white races living in tropical Queensland. His results exhibit no marked variations from the averages obtained in temperate climates.

METHODS.

In all cases analyses of the urine were made for seven—in most cases consecutive—days. Care was taken to prevent decomposition of the specimens by the addition of 2 cc. of 5 per cent. solution of thymol in chloroform.

Kjeldahl's method was employed for estimation of the total nitrogen. Benedict's method for urea, Malfatti's method for ammonia, Johnson's method for creatinine, Salkowski's method for purine bases, Ludwig-Salkowski's method for uric acid, Folin's method for inorganic and ethereal sulphates, Volhard-Arnold's method for chlorides, the uranium acetate method for phosphates, and Folin's method for the total acidity.

Table I. *Statistics regarding Subjects under Observation.*

Subject	Race	Birthplace	Age in years	Height in inches	Weight in kilos.	Years resident in Malaya	Years resident in Singapore
Brahmin	Tamil	Ceylon	23	63	46.1	4.0	3.5
Chinese	Chinese	Singapore	22	62	41.3	22.0	22.0
Tamil	Tamil	Ceylon	28	62	61.7	9.0	4.0
European	British	Australia	35	67	55.6	5.0	5.0
Hindoo	Tamil	Ceylon	23	62	46.9	6.5	2.5
Malay	Malay	Malaya	20	63	56.5	20.0	6.0
Eurasian	Eurasian	Malaya	20	61	49.5	20.0	2.0
Bengali	Bengali	Bengal	18	68	59.1	17.0	3.0
Sikh	Sikh	Punjab	23	67	48.2	12.0	3.0

The composition of the foods and their heat values were taken from a standard work [Leach 1911].

Statistics regarding the subjects are given in Table I. Table II shows the average figures for the nitrogen partition, and Table III the average figures for the non-nitrogenous excretion.

PRESENT OBSERVATIONS.

Brahmin. This man was a vegetarian, his diet consisting of bread, butter, fruit, vegetables, rice (the staple), milk, jam, pulses, tea, coffee, coëco, sugar, salt and spices. He was under observation for three years, and although his diet varied considerably in quantity, it did not vary in quality. The daily total nitrogen output for three years varied from 4.3 g. to 8 g. He lost 4 lbs. weight in three years.

He lived in the school hostel at the time the observations here recorded were made, his average daily diet containing protein 54 g., fat 37, carbohydrate 288, the heat value being 1746 kilocalories, and the daily total nitrogen output averaging 5.1 g. (Table II). The ammonia nitrogen and creatinine nitrogen were not so reduced in quantity as the total nitrogen, urea nitrogen and uric acid nitrogen, when compared with the standard for Europe which is shown in Table II. Therefore the percentages of ammonia nitrogen and creatinine nitrogen were higher than the standard percentages, whilst the percentages of urea nitrogen and uric acid nitrogen were not so greatly altered.

Hindoo. This man partook of the same diet as the Brahmin. He belonged to the same race—Tamil. His average daily diet during the week of observation contained protein 63 g., fat 32, carbohydrate 354, the heat value being 2007 kilocalories. His figures for nitrogen partition, on the whole, confirmed the results for the Brahmin. The average daily total nitrogen output was 5.97 g. representing 37.3 g. protein. The ammonia nitrogen and the creatinine nitrogen suffered a smaller reduction in quantity than the other nitrogenous excretions and were increased in percentage when compared with the standard for Europe (Table II).

Chinese. This student was under observation for three years. His diet

was mixed, containing rice (the staple), bread, butter, milk, jam, beef, mutton, pork, chicken, fish, eggs, vegetables, fruit, sugar, salt, tea, coffee and spices. During six months of observation his daily diet averaged protein 60 g., fat 43, carbohydrate 227, the heat value being 1577 kilocalories and the average daily nitrogen excretion 9.25 g. [Campbell 1917]. He gained 3 lbs. weight in three years.

For the week in which the present observations were made the average daily nitrogen output was 8.60 g. (Table II). When compared with the standard for Europe, the nitrogen excretions, with the exception of the ammonia nitrogen and creatinine nitrogen, were much reduced in quantity, so that the percentages of ammonia nitrogen and creatinine nitrogen were higher than the standard for Europe.

European. This lecturer was under observation for five years in Singapore. During the first three years he lost 14 lbs. in weight; during the last two years his weight was fairly constant. With the loss of weight there was a fall in the diet. The nitrogen output fell from 15 g. to 12 g. per diem. He used an ordinary mixed diet, practically identical in type with the diet in Europe.

The average daily diet during the week of observation consisted of protein 80 g., fat 59, carbohydrates 216, the heat value being 1763 kilocalories, and the average nitrogen excretion 11.70 g. (Table II), which accounted for 73.1 g. protein. The ammonia nitrogen, 0.69 g., was higher than the standard. All the other nitrogenous substances were excreted in smaller quantities than the standard for Europe. The creatinine nitrogen 0.47 g. was not greatly reduced in amount.

Malay, Eurasian, Tamil, Bengali. These four students are considered together because they sat at the same table in the school hostel, and used the same mixed diet, resembling in substance that taken by the Chinese student. The Malay and the Tamil had been under observation since 1916. On the hostel diet the Tamil lost 1 lb. and the Malay gained 5 lbs. in weight in three years. The Tamil's daily diet for three months averaged protein 58 g., fat 32, carbohydrate 277, the heat value being 1672 kilocalories and the total nitrogen excretion 8.24 g. per diem. The Malay's diet was similar, averaging protein 57 g., fat 31, carbohydrate 239 per diem [Campbell 1917].

In the present series of analyses the average daily nitrogen output was 8.74 g. for the Malay, 9.34 for the Tamil, 9.89 for the Eurasian and 7.57 for the Bengali (Table II). In all cases the nitrogenous excretions were much smaller in amount than those of the standard for Europe, with the exception of the ammonia and creatinine. In all subjects the ammonia nitrogen was excreted in greater amount and the creatinine nitrogen in somewhat smaller amount than the standard.

Sikh. This student used a mixed diet similar to that taken by the four students mentioned above, but he lived outside the school hostel. His average daily nitrogen output was 8.64 g. (Table II), the nitrogen partition resembling that of the Malay.

Table II. *Nitrogen Partition.* (Average figures.)

Subject	Diet	Total N		Urea N		Ammonia N		Creatinine N		Uric acid N		Purine bases N		Total N per kilo of body weight
		g.	%	g.	%	g.	%	g.	%	g.	%	g.	%	
Brahmin	Vegetable	5.10	76.53	3.90	5.50	0.50	9.88	0.40	7.99	0.68	1.63	0.013	3.66	0.110
Chinese	Mixed	8.60	81.63	7.02	0.54	6.28	6.72	0.36	4.26	0.14	1.63	0.008	6.11	0.208
Tamil	"	9.34	82.99	7.67	0.62	6.72	6.72	0.42	4.52	0.16	1.75	0.015	4.76	0.151
European	"	11.70	83.59	9.78	0.69	5.96	5.96	0.47	4.05	0.16	1.37	0.016	4.89	0.210
Hindoo	Vegetable	5.97	79.60	4.75	0.39	6.60	6.60	0.38	6.36	0.09	1.62	0.009	5.67	0.127
Malay	Mixed	8.74	78.91	6.89	0.69	7.91	7.91	0.44	5.08	0.17	1.91	0.013	6.05	0.154
Eurasian	"	9.89	80.75	7.98	0.58	5.86	5.86	0.50	5.06	0.14	1.45	0.009	6.79	0.169
Bengali	"	7.57	80.37	6.08	0.63	8.33	8.33	0.45	5.92	0.13	1.76	0.017	3.40	0.128
Sikh	"	8.64	81.45	7.03	0.67	7.76	7.76	0.43	4.97	0.11	1.31	0.021	4.27	0.179
Standard for Europe	"	16.00	85.00	13.60	0.53	3.30	3.30	0.57	3.60	0.25	1.56	0.025	6.39	0.200

CONCLUSIONS.

On the various diets, vegetarian and mixed, used by the Singapore subjects, the nitrogen partition differed from the standard for Europe in the high percentages of ammonia nitrogen and creatinine nitrogen. As regards creatinine, the percentage was high because creatinine, being mainly of endogenous origin, is not greatly reduced in absolute quantity when the total nitrogen is reduced. It will be shown that, in most cases, the amount of ammonia nitrogen was excessive.

Race had no influence on the nitrogen partition, the figures for the different races resembling one another fairly closely, after making due allowance for differences in diets.

Nitrogenous Excretions.

Total Nitrogen. In all cases the daily total nitrogen was much lower than that of the standard for Europe; but if we consider the total nitrogen per kilogram of body weight it will be observed that the figures 0.210 g. for the European, 0.208 for the Chinese, 0.199 for the Eurasian, resembled the standard 0.200 (Table II). The figures for the Brahmin 0.110 and for the Hindoo 0.127 were the lowest mainly because the vegetable protein eaten by them was not so easily digested as the flesh protein taken by the other subjects. The vegetarians used quite as much protein in their food as many of the other subjects. Most of the subjects used about half the standard quantity of protein. McCay, Chittenden and others have already pointed out that it is possible to maintain protein equilibrium on less than half the standard for Europe. The Brahmin and the Hindoo, who had the two lowest figures, were the best students of their years. However, the average Singapore student under observation had not the physique and energy of the average student in Europe nor of the average European resident in Singapore. This supports McCay's views [1910], that races who live on diets of high protein content have better physique and energy than those whose diet contains less protein.

Urea Nitrogen. The text-books state that the urea nitrogen, 16 g., amounts to about 85 per cent. of the total nitrogen excreted, both varying directly with the amount of protein absorbed. According to Folin, when the total nitrogen output falls to 7—8 g., the urea nitrogen is reduced to about 79 per cent. My observations are in general agreement with these conclusions.

Ammonia Nitrogen. In all cases the percentages of ammonia nitrogen were higher than the standard, in some cases very much higher. At first sight this might be expected, as Folin found that the ammonia nitrogen forms about 5 per cent. of the total nitrogen, if the total nitrogen is only 7 or 8 g. Moreover, Malfatti's method of ammonia estimation, which I employed, gives higher results than Folin's method because the former includes also the ammonia of any amino-acids in the urine. Making liberal allowance for the

ammonia of the amino-acids, the ammonia nitrogen may form 6 per cent. of the total nitrogen, if the latter is only 7 or 8 g. and if Malfatti's method is used. But in many cases my percentages were much higher than this; and the absolute quantities of ammonia were also higher than the standard, except in the two vegetarians, Brahmin and Hindoo (Table II); even in them the amounts were higher than one would reasonably expect on their diet. In my previous researches on Singapore medical students [1917] and on Singapore labourers [1918] large amounts of ammonia were detected on many occasions.

Normally the absolute amounts of urea and ammonia rise and fall together with the total nitrogen. In most of my subjects there was a fall in the amounts of total nitrogen and urea nitrogen but a rise in the ammonia nitrogen, which condition indicates an acidosis. What was the cause? Taking all the diets into consideration there was nothing in the food to account for the increase of ammonia. Again the analyst's reports showed that the Singapore water was particularly pure. It is held that the sole use of the urinary ammonia is for neutralisation of acids formed during metabolism. Probably in the Singapore subjects there was a disturbance in metabolism. The severe climatic conditions in Singapore may be responsible, seeing that so many different subjects were affected. Singapore climate is noted for the lassitude it produces. Eight months change in a temperate climate is taken every four years, a more frequent change being usual for women. Further experiments are required to determine whether the acidosis explains any of the discomforts and ill-effects of the climate. Young [1915] working in Townsville, Queensland, has not found any increase in the ammonia nitrogen, but he has noted an increase in the neutral sulphur, which he suggests may be due to an increased tissue metabolism. Townsville and Singapore climates are not very much alike. Singapore, which is much nearer the equator, does not enjoy a cool season, the temperature averaging 80° F. for the whole year; nor does the Townsville climate affect the health of Europeans in the same degree as that of Singapore.

It is well known that fever—that is, a rise of body temperature—causes an increase in urinary ammonia. Hot, moist and still air in Singapore produces great discomfort and a rise of body temperature [Campbell 1919]. In the cotton sheds in England a rise of mouth temperature is observed when the wet bulb thermometer exceeds 75° F.—[quoted by O'Connell 1913]. In Singapore this wet bulb reading is often exceeded for weeks at a time. Probably this caused the acidosis observed by me.

Creatinine Nitrogen. The creatinine coefficients (that is milligrams of creatinine per kilogram of body weight) of my series resembled the standard coefficient for Europe, which ranges from 7—11. My figures varied from 6.8 for the Tamil to 10.1 for the Eurasian (Table II). The Brahmin and the Hindoo, on a diet containing neither creatine nor creatinine, had practically the same coefficient as the subjects on a diet containing these substances.

Obviously all the creatinine of the former subjects was endogenous. The percentages of creatinine were higher than the standard because creatinine, being mainly of endogenous origin, is not lowered in quantity when the total nitrogen is decreased.

Uric Acid Nitrogen. The quantities obtained were all lower than the standard figures, but the percentages agreed with the standard. This was to be expected since uric acid is decreased by cutting down the protein (nucleo-protein) of the diet. The decrease in quantity was particularly noticeable in the case of the Brahmin and the Hindoo, who used a vegetable diet. Most of their uric acid was evidently endogenous.

Purine Nitrogen. None of the subjects excreted so large a quantity as the standard, because they did not consume so large a quantity of the purine bases contained in tea and coffee as the average man in Europe. The tea taken by the Singapore European was a very weak infusion, and he never drank coffee.

Non-Nitrogenous Excretions.

Phosphates. The figures varied from 1.6 g. for the European to 0.9 g. for the Bengali (Table III). The ratio of phosphoric oxide to nitrogen is given in text-books as 1 to 5 or 6; that is about 2.5 g. P_2O_5 . With the exception of the Brahmin and the Hindoo where the ratio was 1 to 5, the average ratio in my results was 1 to 7.5. The Singapore diets evidently contained smaller quantities of absorbable phosphate than the standard diet for Europe.

Table III. *Non-nitrogenous excretion.* (Average figures.)

Subject	Volume in cc.	Specific gravity	Acidity in cc. N/10 NaOH	P_2O_5	NaCl	Inorganic SO_3 g.	Ethereal SO_3 g.
Brahmin	1207	1.013	194	1.1	8.9	0.541	0.097
Chinese	1113	1.014	285	1.3	7.5	1.250	0.107
Tamil	997	1.017	348	1.1	4.9	1.174	0.117
European	1102	1.017	375	1.6	5.4	1.416	0.108
Hindoo	1016	1.020	262	1.1	8.5	0.736	0.147
Malay	1006	1.017	260	1.3	7.0	0.957	0.129
Eurasian	1056	1.017	286	1.3	5.7	1.174	0.085
Bengali	1328	1.009	188	0.9	3.2	0.829	0.102
Sikh	593	1.024	332	1.3	3.0	1.056	0.092

Sulphates. Only the inorganic and ethereal sulphates were estimated owing to the failure to obtain the necessary reagents, etc., for the estimations of the neutral and total sulphur.

The inorganic sulphates weigh about 2.5 g. in the standard for Europe and arise chiefly from the oxidation of the sulphur of protein. In my observations the amounts of inorganic sulphate followed closely the variations of the total nitrogen, the average figures ranging from 0.541 g. for the Brahmin to 1.416 for the European, the total nitrogen figures being 5.1 and 11.7 respectively.

The ethereal sulphates, which have their origin mainly in putrefaction taking place in the intestine, varied considerably in my observations. When present in relatively large amounts, constipation was present and a purgative readily reduced the quantity. High proportions were observed in the vegetarians, the Brahmin and the Hindoo.

Chlorides. The standard figure for Europe is 11 g. In my series the average amounts varied from 3 g. for the Sikh to 8.9 for the Brahmin. The latter and the Hindoo excreted the greatest quantities, probably owing to the large amounts of potassium salts in their diet. Bunge has shown that potassium salts deprive the blood of its chloride, whence the craving for more on a vegetable diet [quoted by Howell 1915].

It is well known that individuals have been kept in good condition on 1 or 2 g. per diem with experimental diets, so that the Sikh's figure was not very low.

Acidity. Calculated in cc. $N/10$ NaOH, the acidity varied from 188 in the Bengali to 375 in the European; or, calculated as HCl, it varied from 0.68 to 1.36 g. The standard figures are given in text-books as 1.5 to 2.3 g. HCl. One would expect the acidity to fall in the Singapore subjects because their diet contained less protein than the standard; and abundant perspiration reduces acidity.

SUMMARY.

1. The manner in which the nitrogen is distributed in the urine of a Brahmin, a Chinese, a Tamil, a European, a Hindoo, a Malay, a Eurasian, a Bengali and a Sikh, all attending the Singapore Medical School, is considered.

2. The absolute quantities of total nitrogen, of urea nitrogen, and of uric acid nitrogen, are much lower than the standard figures for Europe, but the percentages of urea nitrogen and uric acid nitrogen do not differ materially from the standard. In the Chinese, the European and the Eurasian the total nitrogen per kilogram of body weight is about the same as the standard; in the other subjects the figures are lower than the standard.

3. The absolute quantity of purine nitrogen is much lower in all subjects except the Sikh, in whom it is slightly lower than the standard. The percentages vary greatly.

4. In all cases the percentage of ammonia nitrogen is increased. In many cases the absolute amount is increased, and in some cases the increase is well marked. This confirms observations on Singapore students and labourers, already published. The diet and the water are not responsible. It is considered that the excess is due to an increase of acid substances in the blood, owing to a disturbance in metabolism, and that the climate of Singapore may be responsible, since so many different subjects, living under such different circumstances and partaking of different diets, are similarly affected.

5. The quantity of creatinine nitrogen is slightly lower than the standard for Europe but the percentage is higher—in some cases much higher—because

the reduction in the creatinine nitrogen is much less than the reduction in the total nitrogen. This supports the theory that creatinine is mainly of endogenous origin and that it is not lowered greatly by reducing the protein intake. The creatinine coefficient for Singapore resembles very closely that given for Europe.

6. Race apart from diet has no influence on nitrogen partition.

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XXVI. CHEMICAL STRUCTURE AND ANTIGENIC SPECIFICITY. A COMPARISON OF THE CRYSTALLINE EGG-ALBUMINS OF THE HEN AND THE DUCK.

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ANY rational theory of immunity involves a conception of the relation between an antigen and its corresponding antibody. The side-chain theory of Ehrlich, to which immunological investigation in the past two decades has largely owed its direction, gave a purely chemical conception of this relation. More recently attempts have been made to explain certain immunity reactions, such as specific precipitation and agglutination, on purely physical lines, as the mutual precipitation of oppositely charged colloids. This latter hypothesis, which would make the properties of an immune serum depend upon the electric charge on its proteins, and enable these to react with any oppositely charged amphoteric colloid, clearly provides no explanation for the specific discrimination which is the characteristic of immunity reactions. There is evidence, on the other hand, to show that, whatever be the exact nature of the relation between antigen and antibody, it is dependent on the stereochemical structure of the molecule of the antigen. Ten Broek [1914] showed that a racemised protein, which Dakin and Dudley [1913] had found to be quite unaffected by proteolytic enzymes, had also lost all power of evoking the production of antibodies. It was immunologically an inert substance.

This observation suggests a possibility of accounting for the hitherto puzzling phenomenon of the antigenic disparity between proteins, which to ordinary methods of physical and chemical investigation seem to be identical. Corresponding proteins from different species often yield, to an ordinary hydrolytic analysis, the same amino-acids in apparently identical proportions; yet an immunity reaction, such as specific precipitation or anaphylaxis, will discriminate clearly between them. Though the amino-acids are the same, however, and present in identical proportions, it is evident that change in the order of their linkage in the molecule makes possible an immense number of variations in its structure. That specific differences, between corresponding proteins from allied species, might be due to such differences in the intimate pattern of the molecule, was indicated by some recent results.

When a protein is racemised as far as possible by Kossel's method, and subsequently subjected to acid hydrolysis, it is found that certain of the amino-acids have partly or wholly retained their natural optical activity. Dakin [1912] gave reason for believing that the amino-acids thus escaping racemisation are those occupying the terminal positions in the peptide chains, of which the protein molecule is built. By this method Dudley and Woodman [1915] were able to obtain a first indication of structural difference between the caseinogen of cow's and sheep's milk. There was clearly a possibility that this type of structural difference, of which the method only gives a partial indication, might account for the existence of antigenic distinction in cases where an explanation was hitherto wanting.

It seemed to be worth while, therefore, to take two such pure similar proteins, with good antigenic properties, and to examine, on the one hand, their structure, with the help of the racemisation method, and to test, on the other hand, the possibility of distinguishing between them by the highly specific anaphylactic reaction. In the case of the proteins we chose for this purpose the crystalline albumins from the eggs of the domestic fowl and the duck. Preliminary experiments, recorded by Dale and Hartley [1916], had given reason to expect that no difference would be detected by the anaphylactic reaction. If this had been confirmed, and if evidence of structural difference had been obtained, the possibility of explaining antigenic specificity along these lines would have been seriously weakened. The outcome of our experiments, however, has been to give definite evidence of difference in the arrangement of the amino-acids, and, with a more careful and extended series of experiments than those of Dale and Hartley [1916], a clear discrimination between the two proteins by the anaphylactic reaction.

The preparation of the pure, crystalline albumins, and the comparison by the chemical method, were carried out by Dakin, whose share in the work was completed many months ago, before his recently published method for separation of amino-acids was available. Samples of the albumins were sent at the same time to Dale, who was prevented, however, by pressure of other work, from undertaking until recently the comparison by means of the anaphylactic reaction, for which he is responsible. It was thought better to hold back the chemical findings until the biological results were available.

CHEMICAL EXAMINATION.

The albumins were prepared from the whites of hens' and ducks' eggs of unquestionable origin. The method employed for the crystallisation of the albumins was that described by Hopkins [1901] and the products were twice crystallised. In the case of the duck albumin the initial crystallisation was induced by adding rather more acetic acid than that necessary for the crystallisation of hen albumin. Rather over 300 g. of each of the purified proteins were dissolved in water and then dialysed so as to remove most of the adhering

ammonium sulphate. The resulting solutions containing 8—10 per cent. of protein were incubated for 23 days at 37° with the addition of the requisite quantity of caustic soda to bring the concentration of alkali up to half normal. At the end of the incubation period, the alkaline mixtures were hydrolysed by boiling with sulphuric acid and the resulting amino-acids were separated according to the usual methods previously employed in experiments on the racemisation of proteins.

The results as to the relative quantitative yields of individual amino-acids from the two proteins are not considered sufficiently accurate for reproduction, but in general it may be said that they appeared remarkably similar, especially as regards the high yield of phenylalanine and arginine, and relatively low yield of histidine, aspartic acid and leucine.

The results follow:

Amino acid	"Racemised" hen albumin	"Racemised" duck albumin	Comments
Alanine (1)	Not racemised	Not racemised	No difference
Valine (2)	Partly racemised	Partly racemised	" "
Leucine (3)	Mostly racemised	Mostly active	<i>A definite difference</i>
Proline (4)	" "	Mostly racemised	No difference
Phenylalanine	Completely inactive	Completely inactive	" "
Tyrosine (5)	Inactive	Inactive	" "
Aspartic acid (6)	Mostly inactive, some active	Completely inactive	<i>Definite difference</i>
Glutamic acid	Completely inactive	" "	No difference
Histidine (7)	" "	Mostly active	<i>Definite difference</i>
Arginine	Active	Active	No difference
Lysine (8)	Inactive	Inactive	" "

(1) The specific rotations for the alanine hydrochlorides were + 6° and + 7.5° respectively compared with a theoretical value of + 10° but this represents no greater racemisation than usually occurs in the separation of the active alanine from proteins. No racemisation of the alanine group in the proteins by the alkali is indicated.

(2) The valine fraction was not obtained perfectly free from leucine and in both cases was mostly racemised.

(3) A marked difference was noted here. The first large fraction of pure leucine (9 g.) crystals was absolutely inactive in the case of the hen albumin while the corresponding fraction (7 g.) from the duck albumin was almost exclusively the *l* form, $[\alpha]_D = + 15^\circ$ in 20 per cent. hydrochloric acid. Theoretical value = + 15.6°. The whole of the leucine from the duck albumin had a mean rotation in hydrochloric acid of + 13.3° indicating about 85 per cent. of the active variety, while the corresponding rotation of the hen albumin leucine was + 4.3°, and this is probably partly due to contaminating valine. Certainly not more than 27 per cent. of the leucine from the racemised hen albumin was in the active form.

(4) Most of the proline from both preparations was inactive. The hen product had a rotation in water of - 5° while the proline from duck egg

albumin showed a value of -9.9° . Pure *l*-proline has a specific rotation in water of -77.4° , and while much racemisation ordinarily occurs in the process of isolation, the above values are much lower than those previously observed even from other racemised proteins, so that it appears that most of the proline groups in both proteins were inactivated by the action of alkali.

(5) No perfectly pure specimens of tyrosine were obtained, since the more soluble inactive acid in small amounts is hard to separate from leucine and other impurities. No laevo-rotation was observed in any case.

(6) The whole of the aspartic acid from the duck egg albumin was optically inactive as well as the mother-liquors from which aspartic acid was separated, so that it is certain that none of the active acid was present. From the hen egg albumin, in addition to a considerable amount of racemic aspartic acid, a small amount (0.3 g.) of the pure laevo-acid was separated. It decomposed around 270° , was faintly laevo-rotatory in alkaline solution, and had $[\alpha]_D^{20} = +24.5^\circ$ in hydrochloric acid solution (3 mols). Titration with standard alkali using litmus as indicator proved the purity of the product and freedom from leucine. Optically pure aspartic acid under similar conditions shows a specific rotation of $+25.7^\circ$. Since in any case *l*-aspartic acid is largely racemised during its separation by the ester method, the detection of a little of the laevo-acid must be taken to indicate the original presence of considerably more of the active acid.

(7) The whole of the histidine fraction from the hen egg albumin was completely inactive both as free base and as salts. On the other hand the histidine from the duck egg albumin, though small in quantity, was strongly laevo-rotatory, having $[\alpha]_D^{20} = -27.2^\circ$, compared with -39° for the optically pure base. 70 per cent. of the histidine was therefore made up of the laevo-variety. In each case the histidine was successively separated and identified as phosphotungstate, silver salt, precipitation with mercuric sulphate, and picrolonate.

(8) In neither case could any dextro-lysine be detected, but it is noteworthy that in each case the lysine fraction was feebly laevo-rotatory. The cause of this is obscure, but it was certainly not due to contamination with proline.

ANAPHYLACTIC REACTIONS.

The experiments were all made on guinea-pigs, each of which received a preparatory injection of 1 mg. of one or other of the albumins. After intervals varying from 19 to 31 days they were tested for sensitiveness. Most of the tests were made, by the now familiar method, on the isolated uterine muscle, young virgin females being chosen for this purpose. The use of this method enabled sensitiveness to both albumins to be tested on the same animal. In most cases some degree of sensitiveness to both was present. In many a high degree of sensitiveness to the albumin not used for the preparatory injection was observed. In all cases, however, a preferential sensitisation

to the antigen given in the preliminary injection could be detected. Even when a small first dose of the non-specific antigen caused an apparently maximal contraction, the uterine muscle could be completely desensitised to this and yet retain sensitiveness to the specific one; whereas desensitisation to the specific antigen left the muscle completely insensitive to the other. In some cases the specificity was apparently absolute, the muscle being indifferent to a large dose of the non-specific and subsequently reacting typically with the specific antigen.

A small confirmatory series of experiments was made on intact animals, the doses being injected into the jugular vein.

The albumins were always dissolved in physiological saline solution, and the greatest care was exercised, by the use of separate sets of measures, pipettes, etc., to avoid any possibility of contaminating one solution with traces of the other.

EXPERIMENTS ON THE ISOLATED UTERUS.

The following are characteristic records.

Expt. 1. Sensitised with 1 mg. of duck albumin. 19th day.

- | | |
|-----------|---|
| 1st horn. | (1) 0.1 mg. Hen. No reaction.
Change Ringer's solution. |
| | (2) 0.1 mg. Duck. Good but not maximal contraction.
Change Ringer. |
| | (3) 1 mg. Hen. Small reaction.
Change Ringer. |
| | (4) 1 mg. Hen. Nil. (Desensitised to hen.) |
| | (5) 1 mg. Duck. Good but not maximal reaction. |
| 2nd horn. | Not tested. |

Expt. 2. Sensitised with 1 mg. hen albumin. 21st day.

- | | |
|------------------------|---|
| 1st horn. | (1) 0.1 mg. Duck. Nil. |
| | (2) 0.1 mg. Hen. Maximal contraction.
Change Ringer. |
| | (3) 0.1 mg. Hen. Nil. |
| 2nd horn (see Fig. 1). | (1) 2 mg. Duck. Nil. |
| | (2) 0.1 mg. Hen. Maximal contraction.
Change Ringer. |
| | (3) 0.1 mg. Hen. Nil. |
| | (4) 1 mg. Hen. Nil. |

This is an example of very perfect specificity.

Expt. 3. Sensitised with 1 mg. hen. 30th day.

- | | |
|-----------|--|
| 1st horn. | (1) 0.1 mg. Duck. Maximal contraction.
Change Ringer. |
| | (2) 0.01 mg. Hen. Weak contraction. |
| | (3) 0.01 mg. Hen. Nil. |
| | (4) 0.1 mg. Hen. Moderate contraction. |
| | (5) 1 mg. Hen. Very weak contraction. |

2nd horn.

- (1) 0.01 mg. Duck. Moderate contraction.
- (2) 0.1 mg. Duck. Nil.
- (3) 1 mg. Duck. Weak contraction.
Change.
- (4) 1 mg. Duck. Nil. (Desensitised to duck.)
- (5) 0.01 mg. Hen. Weak contraction.
- (6) 0.1 mg. Hen. Moderate contraction.
Change Ringer.
- (7) 1 mg. Duck. Nil.
Change Ringer.
- (8) 1 mg. Hen. Moderate contraction.

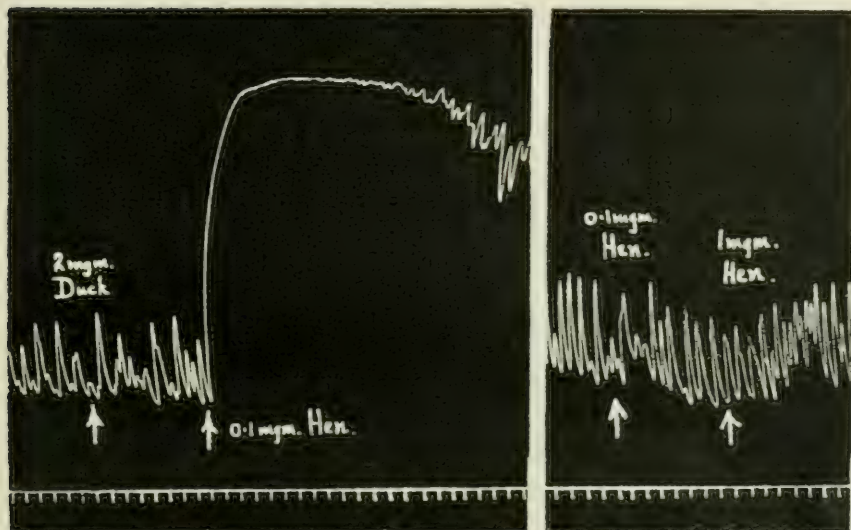


Fig. 1. See Expt. 2, 2nd horn. Sensitised with 1 mg. of Hen albumin, 21st day.

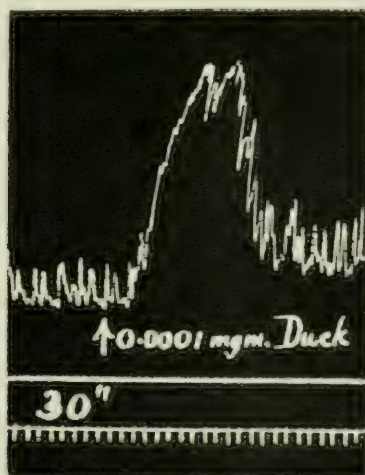


Fig. 2. See Expt. 4, 2nd horn. Effect of 1 part of specific antigen (Duck albumin) in 500 millions of Ringer's solution.

The maximal response given to the first dose might easily give the impression that the sensitisation to duck albumin was as intense as to hen. The record of the second horn, however, shows clearly that complete desensitisation to duck leaves a power of responding even to 0.01 mg. of hen albumin. The same distinction is shown in the next record, in which the non-specific sensitisation is of a high order, but that to the specific antigen is much greater. It may be noted that the bath containing the organ held 50 cc., so that a dose of 0.0001 mg. produces a concentration of only 1 in 500 millions.

Expt. 4. Sensitised with 1 mg. duck. 31st day.

- 1st horn. (1) 0.01 mg. Hen. Maximal contraction.
Change Ringer.
(2) 0.01 mg. Hen. Very small contraction.
Change Ringer.
(3) 0.01 mg. Hen. Nil.
(4) 0.01 mg. Duck. Moderate contraction.
- 2nd horn. (1) 0.0001 mg. Duck. Moderate contraction. (See Fig. 2.)
Change Ringer.
(2) 0.001 mg. Duck. Nil.
(3) 0.01 mg. Duck. Maximal contraction.
Change.
(4) 0.1 mg. Hen. Nil.

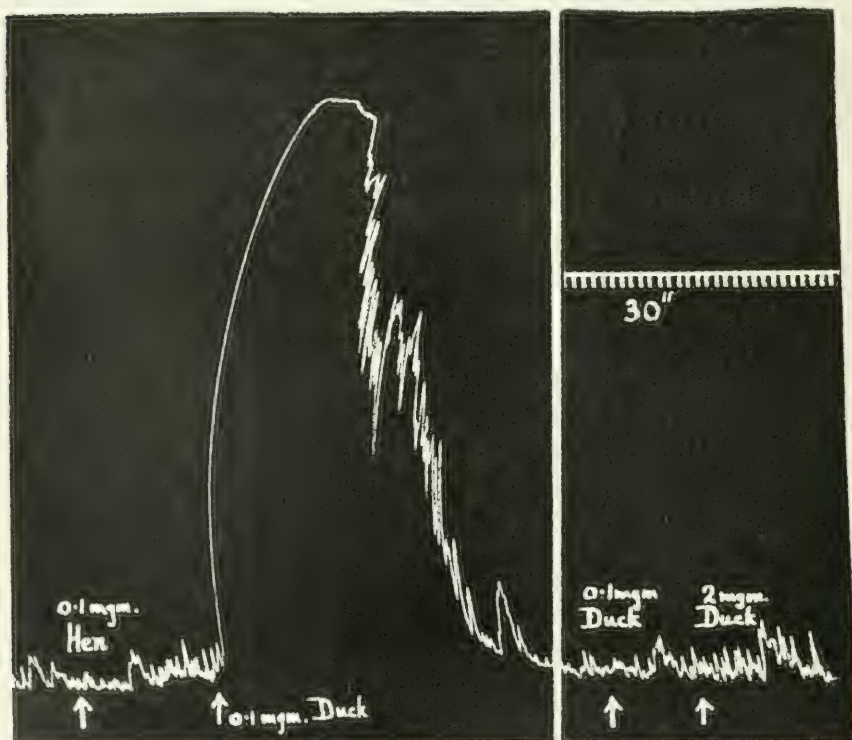


Fig. 3. See Exp. 6, 1st horn. Sensitised with 1 mg. of Duck albumin, 28th day.

Expt. 5. Sensitised with 1 mg. duck. 30th day.

- 1st horn. (1) 0.001 mg. Hen. Nil.
 (2) 0.001 mg. Duck. Moderate contraction.
 Change Ringer.
 (3) 0.1 mg. Hen. Nil.
 (4) 0.1 mg. Duck. Larger contraction than at (2). Not maximal.
- 2nd horn. Not tested.

Expt. 6. Sensitised with 1 mg. duck. 28th day.

- 1st horn. (See Fig. 3.) (1) 0.1 mg. Hen. Nil.
 (2) 0.1 mg. Duck. Maximal contraction.
 Change Ringer.
 (3) 0.1 mg. Duck. Nil.
 (4) 2 mg. Duck. Nil.
- 2nd horn (see Fig. 4): (1) 2 mg. Hen. Moderate contraction.
 Change Ringer.
 (2) 0.1 mg. Duck. Maximal contraction.
 Change Ringer.
 (3) 0.1 mg. Duck.
 (4) 2 mg. Duck.

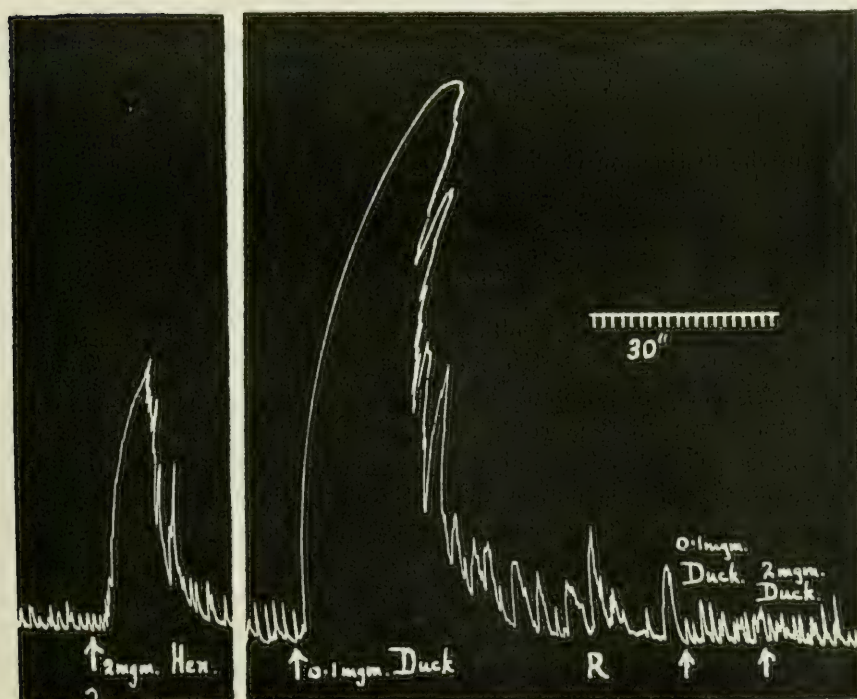


Fig. 4. Continuation of Expt. 6. 2nd horn:

EXPERIMENTS ON INTACT GUINEA-PIGS.

Sensitising injections hypodermic; test injections intravenous.

1. Sensitisation—1 mg. hen albumin.

Weight in g.	Days after first injection	Second dose	Result
300	32	0.1 mg. Hen	† in 4'
290	30	0.05 Hen	† in 4'
200	30	0.01 Hen	Slight symptoms
180	33	0.01 Hen	Slight symptoms
270	32	0.1 Duck	Moderate symptoms
245	30	0.5 Duck	Slight symptoms

The lethal dose for hen albumin apparently lies between 0.01 and 0.05 mg. For duck albumin it is not reached even at 0.5 mg. though there are symptoms of reaction even with 0.1 mg.

2. Sensitisation—1 mg. duck albumin.

Weight in g.	Days after first injection	Second dose	Result
290	32	0.1 mg. Duck	† in 3'
250	30	0.05 Duck	† in 3'
225	30	0.01 Duck	Slight symptoms
200	33	0.01 Duck	Slight symptoms
290	32	0.1 Hen	† in 3'
240	30	0.05 Hen	Nil

Again the distinction is clear, but a guinea-pig dies, in this series, after 0.1 mg. of the non-specific albumin.

DISCUSSION.

So far as they go, the results support the conception that the stereochemical structure of the protein molecule is at least an important factor in antigenic specificity. Without the information given by the racemisation method these two proteins would have been indistinguishable, except by an immunological test. The racemisation having shown a structural difference, the presumption that structure and antigenic specificity are related seems to be warranted. This conclusion, however, provides only a first step towards a conception of the relation between antigen and antibody. In due course it will be of interest to discover whether that protein of an immune serum, which carries the specific "antibody" action, shows any difference of molecular pattern from the corresponding protein of a normal serum. Meanwhile the only kind of relation which seems to provide even a distant analogy is that of enzyme and substrate; but the discrimination of the antibody is far more delicately specific.

SUMMARY.

The crystalline albumins from the eggs of the domestic fowl and duck behave as distinct antigens for the anaphylactic reaction. This difference corresponds with a difference in structure, as revealed by the fact that, when the proteins are racemised, the amino-acids escaping racemisation are not identical in the two cases.

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XXVII. THE RÔLE OF THE PLASMA PROTEINS IN DIFFUSION.

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THERE is nothing more remarkable than the maintenance of a constant composition of the blood plasma under the most varied conditions. This is shown very clearly in the regulation of the reaction of the blood, and attention has now been directed by Haldane and Priestley [1916] to a similar control as regards the water and salts when large quantities of water or salt solutions are ingested or in the opposite condition of profuse sweating. The electrolyte concentration of the serum was shown by Priestley [1916] to be very slightly diminished when large quantities of water were taken and slightly increased when salts were added to the water. Again, after severe loss of blood, the volume of the circulating fluid is rapidly restored by the entrance of fluid from the tissue spaces and, as will be shown later, this fluid which enters must possess approximately the electrolyte concentration of the original blood plasma. Under these varied conditions there is one class of constituent of the plasma, as there is also one in the tissues, which can only with great difficulty leave its original situation and that is the colloidal element, the protein. Thus after the entrance of non-protein-holding fluids into the blood by ingestion or injection, the colloidal concentration is lowered and on removal of a similar fluid, as in sweating, it is raised. Again after haemorrhage the fluid which enters being either free from or poor in colloid leaves the colloidal concentration at a lower level than the normal. Variations in the colloidal concentration of the blood are therefore dependent upon the addition or withdrawal of non-colloidal solutions. Neither the proteins of the tissue fluids nor the proteins of the plasma can be readily transported, and the exchange of water and salts from one side to the other must be largely governed by the colloidal concentration on these two sides. To Starling and others we owe much of our knowledge of one way in which the protein concentration of a fluid may make itself felt, namely by affecting the osmotic pressure level and so controlling the filtration process through a membrane impermeable for the protein molecule. Before entering upon the consideration of the various ways in which the protein content of a fluid may affect saline diffusion, a brief reference will be made to certain changes which the blood plasma

undergoes after haemorrhage, in so far as these may contribute to our knowledge of the nature of the exchange between tissue fluids and blood. Many investigations have been carried out dealing with this subject and that there are certain well defined changes has been clearly established. After haemorrhage the blood shows a decrease in specific gravity, viscosity, and protein content. The chloride concentration is little if at all affected, while according to Taylor and Lewis [1915] the non-protein nitrogen and urea are apparently increased. Special reference however requires to be made to the degree of the alterations in specific gravity, viscosity, protein and electrolyte concentration with a view to the subsequent consideration of the part played by protein and salts in the exchange between blood and tissues. The effects of haemorrhage therefore were investigated in order to determine the nature and degree of the changes produced in the plasma.

EFFECTS OF HAEMORRHAGE.

The methods employed in the determination of these changes were as follows. The specific gravity was estimated in the usual way, a tube of the Sprengel type being employed and the tube filled with water and then with plasma at 18°.

Viscosity was determined by the Ostwald type of viscosimeter and determinations made either at 25° or 37°.

Conductivity was determined by the Kohlrausch method, the conductivity cell used being of the Washburn type and the observations were made in electrically regulated thermostats at 25° and 37°.

Nitrogen was estimated by Kjeldahl's method.

After loss of blood varying from 30—57 per cent. of its total volume, in both the cat and dog, the specific gravity, viscosity and percentage of nitrogen fall while the conductivity rises. The fall in specific gravity is very slight, the mean percentage fall in the cat being 0.2 and in the dog 0.13. The fall in viscosity is more distinct, ranging on an average from a 5 per cent. decrease in the viscosity coefficient in the dog to 10 per cent. in the cat. The nitrogen percentage shows a mean decrease of about 14.5 in the cat to 18 in the dog. The rise in conductivity, although always to be observed, is comparatively small, ranging from 1.92 mean percentage rise in the dog to 3.8 in the cat. These changes are given in detail in the following table (Table I).

The decrease in the nitrogen content of the blood after haemorrhage and the fall in viscosity are the most distinctive alterations, and the latter is the expression of the lowered protein content, as the non-protein nitrogen is evidently increased as a result of haemorrhage. The fluid which enters the blood after haemorrhage must have at least the electrolyte concentration of the normal plasma because even after the most severe loss of blood there is no evidence of a fall in conductivity. It is therefore evident that the blood plasma tends to maintain its normal electrolyte concentration not only after

Table I. *Effects of haemorrhage on blood plasma.*

	Sp. gr.	% fall	Viscosity	$K \times 10^4$ at 37°	% rise	Nitrogen %	% fall
Cat (2.23 kgrms.)							
Before haemorrhage	1.0275	—	1.751 (η 25°)	—	—	—	—
40 minutes after 60 cc. loss	1.0249	0.25	1.545 "	11.7	3.1	—	—
Approximate 30 % loss							
Cat (2.6 kgrms.)							
Before haemorrhage	1.0300	—	1.840 "	—	—	1.064	—
60 minutes after 120 cc. loss	1.0281	0.18	1.668 "	9.3	—	0.927	12.8
90 " " "	1.0272	0.27	1.529 "	16.9	6.2	0.915	14.0
Approximate 57 % loss							
Cat (2.15 kgrms.)							
Before haemorrhage	1.0265	—	1.604 (η 37°)	—	—	1.033	—
75 minutes after 50 cc. loss	1.0251	0.13	1.521 "	5.1	2.1	0.858	16.9
Approximate 43 % loss							
Dog (18 kgrms.)							
Before haemorrhage	1.0249	—	1.593 (η 25°)	—	—	0.935	—
3 hours after 500 cc. loss	1.0236	0.12	1.467 "	7.9	2.5	0.806	13.7
Approximate 34 % loss							
Dog (13 kgrms.)							
Before haemorrhage	1.0250	—	1.409 (η 37°)	—	—	0.860	—
40 minutes after 550 cc. loss	1.0237	0.12	1.359 "	3.5	2.6	0.691	19.6
2 hours after 550 cc. loss	1.0232	0.17	1.342 "	4.7	.66	0.662	23.0
Approximate 52 % loss							

the ingestion of fluids but also after severe loss of blood from the body. As to the nature of the salts entering the blood vascular system, from a small number of analyses it was evident that at least the chloride content of the plasma was not distinctly altered. From Luckhardt's [1910] observations the chloride content of the lymph and tissue fluids is rather higher than that of the blood serum in the same animal. The passage of such a fluid from tissue spaces into blood must affect the protein-salt equilibrium between tissue elements and the fluid which bathes them. It was therefore thought advisable to study by as simple methods as possible the influence of proteins on salt diffusion.

DIFFUSION OF NaCl FROM PROTEIN SOLUTIONS.

In order to study the rôle of proteins in saline diffusion attention was particularly directed to the velocity of diffusion under as nearly as possible constant conditions. The influence of proteins on the diffusion of salt from a protein salt solution into water was investigated in one or other of the following ways. The solutions were separated from one another either by means of a collodion membrane in tubular form, or as a flat diaphragm. The rate of change was followed by making conductivity determinations and also by analysing the saline content of the water at regular intervals. The water towards which the salt was diffusing was either kept running at a constant slow rate or it was changed at stated intervals in order that the concentration gradient should be fixed by the saline concentration in the protein salt solution and therefore be a function of the same. In order that the experimental conditions should be tested as regards their suitability for determining velocity coefficients, the rate of diffusion from a simple aqueous solution of the salt in the same concentration and under exactly the same conditions, as regards filtering membrane, rate of change of water, temperature, etc., was always determined either before or after, or both before and after, the corresponding experiment with the protein salt solution.

All experiments were carried out in one or other of two thermostats heated by electric radiators and with suitable toluene-mercury regulators.

One thermostat was kept at a constant temperature of 25°, the other at 37°, or in some cases slightly over that temperature.

The collodion membranes were made by a modification of Walpole's method [1915], the films being allowed to dry in a closed chamber saturated at 20° with alcohol-ether vapour instead of being freely exposed to air. By this method the passage of the protein through the membrane was diminished to a mere trace.

Conductivity determinations were made by the ordinary Kohlrausch method, the platinised plates being fixed in a definite position in the collodion tube. In the case of the diaphragm dialyser, two glass vessels of the small desiccator cover form (300 cc. capacity) with flanges were used, the collodion

diaphragm with a diffusing surface of 34 sq. cm. being clamped between the two vessels and the edges of the flanges carefully sealed with Chatterton's cement. Tests were always carried out before each experiment in order to exclude any possibility of leakage at the margin. The glass stopper bearing the platinised plates could be placed in either of the two chambers. The majority of the conductivity determinations were made in the fluid from which the salt was diffusing out. The chloride estimations were made by Volhard's method, and in cases where protein was present the modification of this method described by Rappleye [1918] was employed. In the first set of experiments, the diffusion rates of salt into conductivity water from (a) simple NaCl solution in water, (b) gum arabic solution with the same salt content, and (c) ox blood serum were investigated. The experiment was carried out in the diaphragm dialyser, the water being changed at the end of each three hour period and the chloride estimated. The conditions of the experiment in all three cases were identical. In each case the solutions were warmed to 37.5° before placing them in their respective chambers, and the conductivity water introduced at the end of each three hour period was also raised to the same temperature before replacing the preceding dialysate. The fall in conductivity in the three saline solutions was determined at frequent intervals and the one hour readings are given in Table II and are plotted out in Fig. 1.

Expt. 1 (Fig. 1). Diffusion from NaCl, NaCl-gum, and serum to water.

- Sol. A. 0.795 per cent. NaCl.
 „ B. 0.795 per cent. NaCl in 6 per cent. gum arabic.
 „ C. Ox blood serum (0.624 per cent. NaCl).
 Temperature 37.5°.

The dialysate was replaced by fresh conductivity water at the end of the third, sixth and ninth hours.

In Fig. 2 the results of the chemical analyses of the chloride content in terms of the percentage NaCl loss during diffusion are plotted; the experiments in each case were for a period of 12 hours.

The viscosity coefficients at 37.5° of the three solutions were

	$\eta_{37.5^\circ}$ (Water=1.)
A	1.014
B before diffusion	2.676
after „	3.080
C	1.878

As may be readily observed on examination of the curves, the rate of passage out of the salt from the protein solution is evidently not a function of the viscosity, as the gum arabic solution is the more viscous fluid.

From about the fifth—sixth hour the velocity of diffusion from the serum becomes distinctly slower than in the case of the gum arabic or the simple salt solution. The diffusion from the gum arabic solution is somewhat slower and also more regular than in the case of the simple salt solution. If one regards the concentration gradient as determined by the salt concentration

in the solution from which the salt is diffusing and that therefore there is little back effect, then according to the law of mass action the rate of diffusion at the time t should be proportional to the concentration at that time. Taking x_0 as the original concentration in each case and x as the concentration at the stated periods, the velocity coefficients for the time unit one hour, determined from the equation

$$k = \frac{1}{t} \log_e \frac{x_0}{x},$$

are shown in Table II.

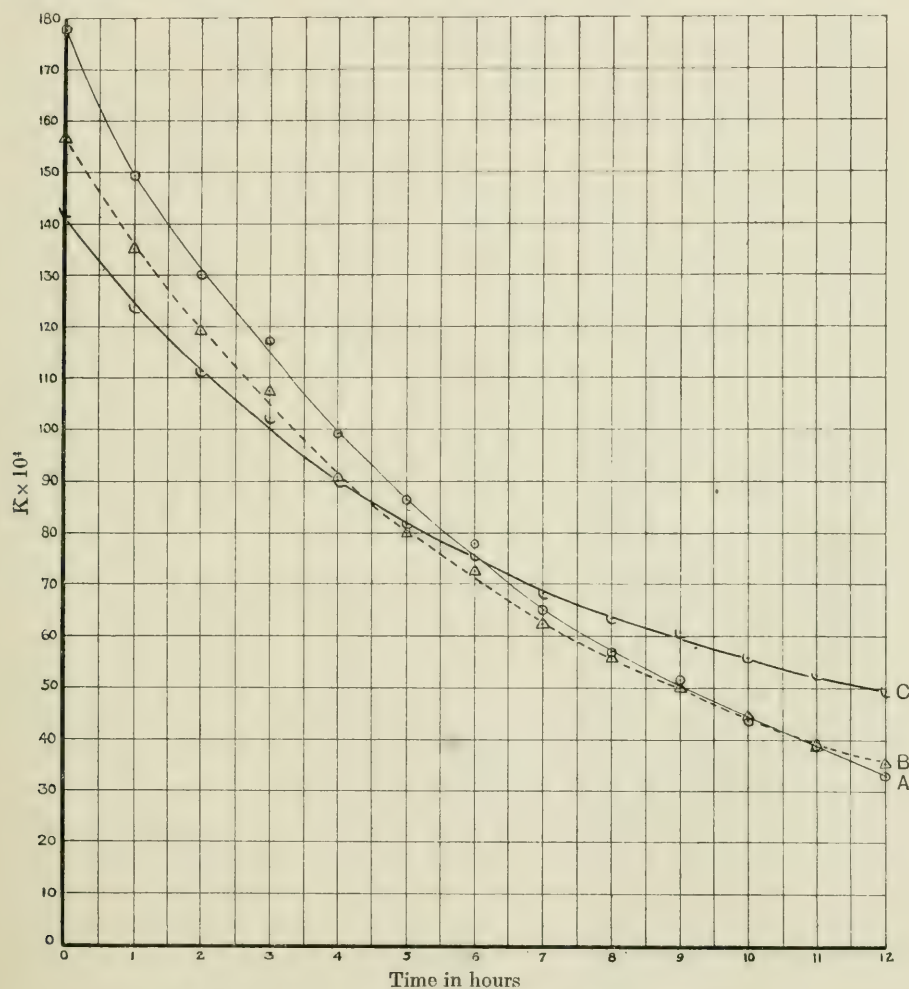


Fig. 1. Diffusion Curves. (Conductivity Measurements).
 0.795 % NaCl against Water. \odot ————— A.
 Gum Arabic solution in .795 % NaCl against Water. \triangle - - - - B.
 Blood Serum against Water. \odot ————— C.

The coefficients so determined are only approximate, but for comparative purposes between the three solutions they are valid.

Table II. *Velocity coefficients of diffusion in the three solutions.*

A.			
Time in hours	NaCl present	$\frac{x_0}{x}$	$k = \frac{1}{t} \log_e \frac{x_0}{x}$
0	1.652 (x_0)	—	—
3	0.986	1.675	0.171
6	0.551	3.000	0.183
9	0.309	5.346	0.186
12	0.131	11.884	0.206

Viscosity $\eta_{37.5^\circ} = 1.0146$.

B.			
0	1.669	—	—
3	1.018	1.639	0.164
6	0.618	2.700	0.165
9	0.378	4.415	0.166
12	0.228	7.320	0.165

Viscosity $\eta_{37.5^\circ} = 2.676$ before and 3.080 after diffusion.

C.			
0	1.560	—	—
3	1.037	1.504	0.136
6	0.720	2.166	0.129
9	0.597	2.613	0.106
12	0.481	3.243	0.098

Viscosity $\eta_{37.5^\circ} = 1.878$.

In the case of blood serum there is a gradual fall in the coefficient, most distinct between the sixth and ninth hours, the gum arabic saline shows a very regular rate of diffusion proportional to the concentration while the simple watery solution of salt shows a rather variable rate under the experimental conditions due to back pressure effects, but the rate is faster at the lower concentrations.

As the gum arabic solution is the most viscous of the three it is evident as already mentioned that some factor other than that of viscosity is concerned in the diffusion of salt from serum. This of course in no way invalidates the experimental evidence in support of the advantage of the addition of gum to saline injections, the value of which is undoubted as shown by the researches of Bayliss [1918] and others. The blood serum differs from the gum arabic solution in possessing a constituent which requires a certain salt concentration for its solution, namely globulin, and it is during the later period of diffusion that this body separates out. The question whether the other protein in solution in the serum plays any part as a governing factor was examined in the case of dialysed horse plasma. The experiment was carried out in a collodion tube. In order that the effect of the globulin might be compared with the globulin-free (or globulin-poor) solution similar diffusion experiments were carried out with normal and dialysed (10 days) plasma.

The globulin suspension was removed from the dialysed plasma by centrifugalisation. The collodion tube was fitted with a rubber stopper through

which passed two glass tubes carrying platinum plates and also two tubes for filling purposes. The tube was then inserted in a glass jacket, the volume of the outer space being 200 cc. and that of the tube 90 cc. The apparatus was placed in a thermostat at 25°. The solution containing the salt, warmed to 25°, was placed in the inner tube and water, previously heated to the same temperature by passing through spiral glass tubing in the bath, was allowed to

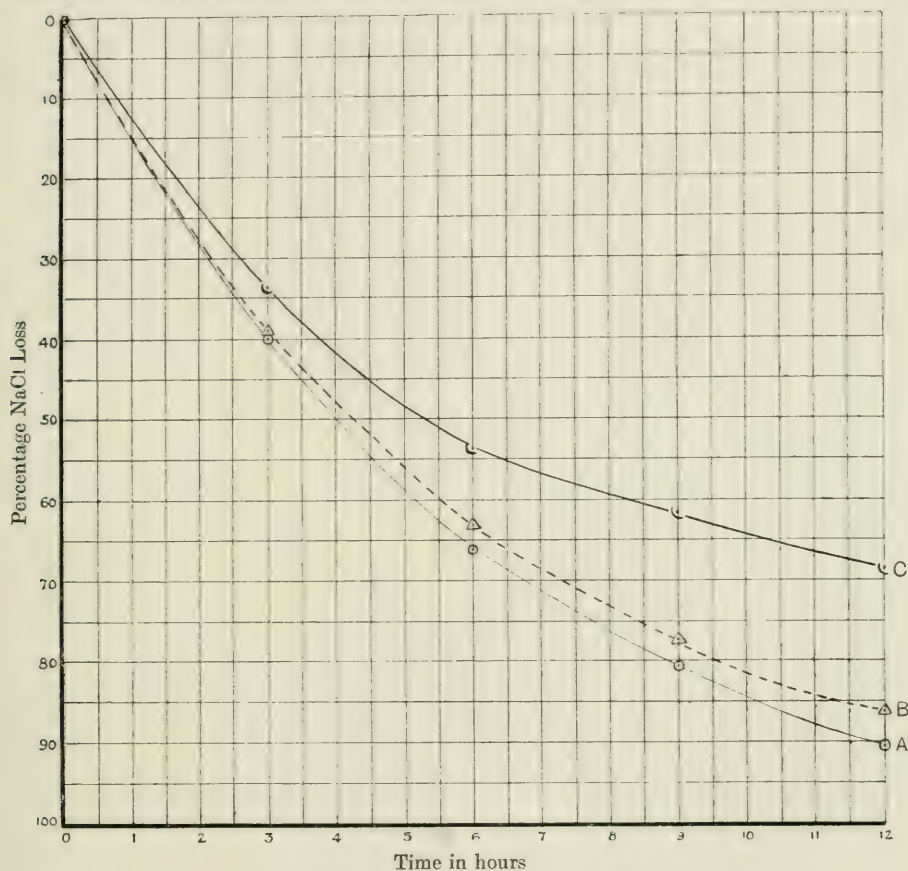


Fig. 2. Diffusion Curves. (Percentage NaCl Loss.)

0.795 % NaCl. ○ ——— A.
 Gum Arabic containing 0.795 % NaCl. △ - - - B.
 Blood Serum. (0.624 % NaCl.) ● ——— C.

flow through the outer jacket at a rate of 250 cc. per hour. The outflow fluid was collected at two hour intervals and the NaCl estimated. The diffusion rates from the following three salt-containing solutions were then determined and are plotted in Fig. 3 as percentage NaCl loss against time.

Expt. 2. Diffusion from NaCl, normal horse plasma, and dialysed horse plasma (with salt).

Sol. A. 0.547 per cent. NaCl in water.
 „ B. Dialysed Plasma containing 0.574 per cent. NaCl.
 „ C. Normal Plasma 0.547 per cent. NaCl.

During the first six hours there is not much difference in the rates of diffusion of NaCl from normal plasma and from the simple watery solution of the salt, although the former shows the slower rate. Diffusion from the dialysed horse plasma is distinctly the slowest of the three. Thus 82.96 per cent. is lost from A, 75.57 per cent. from B, and 79.05 per cent. from C.

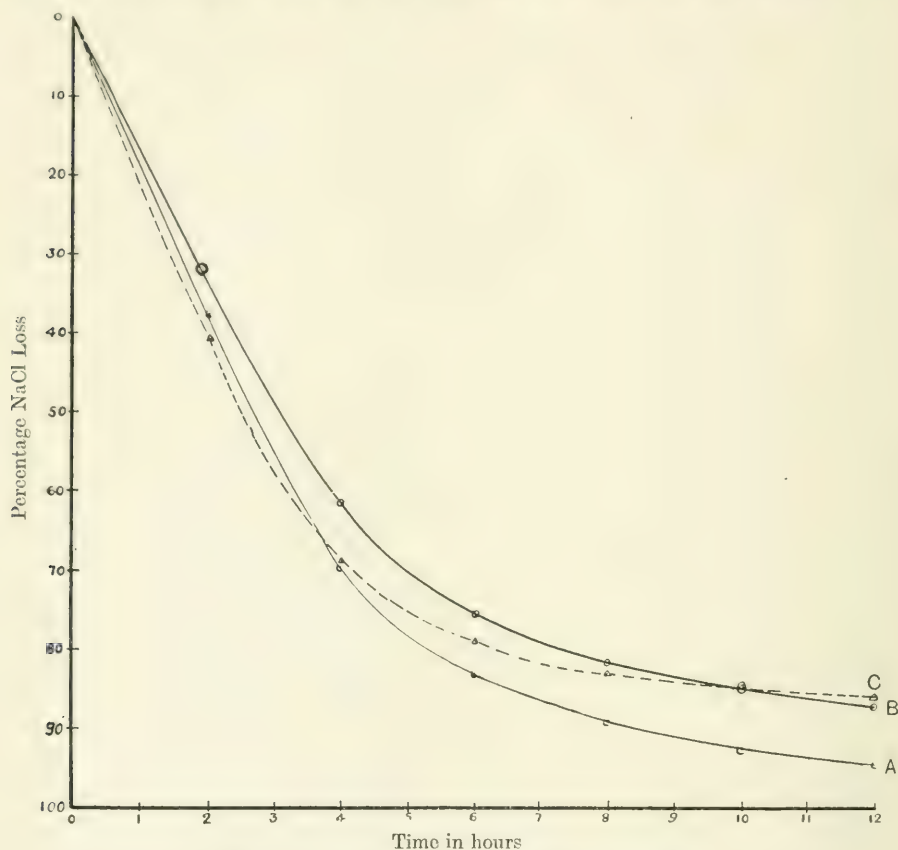


Fig. 3. Diffusion Curves. (Percentage NaCl Loss.)
 NaCl Solution \circ ————— % NaCl=0.547; A
 Dialysed Plasma \odot ————— „ =0.574; B.
 Normal Horse Plasma Δ - - - - - „ =0.547; C.

In the last six hours however there is a distinct difference between the globulin-free (or poor) solution and the globulin-containing one, the rate of diffusion being slower in the latter than in the former.

If one compares the velocity coefficients of the first four hours and the last eight hours in each of the three solutions, this becomes evident.

	A	B	C
k 1st 4 hours : k last 8 hours	1 : 1.08	1 : 0.92	1 : 0.78

Fig. 3 shows clearly that the curve of diffusion in the case of normal horse plasma begins to show the slowing in the diffusion rate about the fourth hour,

and as diffusion from the dialysed plasma is distinctly slower than from the normal in the preceding four hours the two curves converge from that point onwards.

It is evident therefore as in Experiment 1 that the globulin-holding solution disturbs the diffusion rate in the later stages and as the globulin-free solution in Experiment 2 does not show the same change, the protein other than globulin is not the main cause of the altered rate of diffusion observed in the later stage. As in the case of the blood serum or plasma the fall in diffusion rate is always accompanied by a separation of the globulin, it was thought advisable to investigate the salt diffusion under conditions where the globulin did not separate out on dialysis. The diffusion rates of salt from dialysed plasma and from globulin in acid solution (prepared from the globulin separated out from the normal plasma) were therefore investigated. During the period of diffusion the acid globulin only showed a faint trace of separation from the mixture, the solution becoming faintly opalescent. The globulin obtained by dialysis for a period of ten days from 200 cc. horse blood plasma, was removed by centrifugalisation, dispersed in 5 cc. 0.1N HCl, and the volume made up to 200 cc. with water. The mixture was allowed to stand for 24 hours at room temperature and the small residue of undissolved globulin was then removed. The solution was made up to 0.470 per cent. NaCl. The nitrogen value of the solution was 0.146 per cent. The rates of diffusion to water from 0.1 normal NaCl in water, 0.1 normal NaCl in dialysed plasma (nitrogen 0.796 per cent.), and 0.470 per cent. NaCl in the acid solution of globulin were determined. To compare with the acid solution of globulin an alkaline solution was prepared in the same way, 6 cc. 0.1N NaOH being added. The nitrogen value of this solution was 0.148 per cent. As the alkali globulin solution on dialysis showed a precipitate it could not be made use of for the purpose of this special experiment, in which the influence of globulin in solution throughout the diffusion process was being investigated. The salt-containing solutions were placed in the collodion tube of the same apparatus, water being allowed to pass through at a rate of 250 cc. per hour, and in addition at two hour intervals the outer fluid was removed and replaced by fresh conductivity water. The water which had run through during the two hour period was added to that drawn off at the end of the period and the chloride content estimated. The results are shown in Fig. 4.

Expt. 3. Diffusion from NaCl, dialysed horse plasma (0.1 normal NaCl), acid globulin (0.470 per cent. NaCl), alkali globulin (0.528 per cent. NaCl) to water.

- Sol. A. NaCl, 0.1 normal.
- „ B. Dialysed Horse Plasma.
- „ C. Acid Globulin.
- „ D. Alkali Globulin.

As is evident from the curves in Fig. 4, the diffusion rate from acid globulin is much slower throughout than from the other three solutions which show

diffusion curves of much the same form. The difference in the diffusion rate of salt from the acid globulin solution is possibly related to the influence of the acid on the hydrolysis of the globulin salt, retarding it to such an extent that the solution at the end was only faintly opalescent. Under those circumstances the globulin-salt compound had not evidently undergone dissociation to the same extent as in the alkaline medium. Hardy [1905]. Pauli [1903].

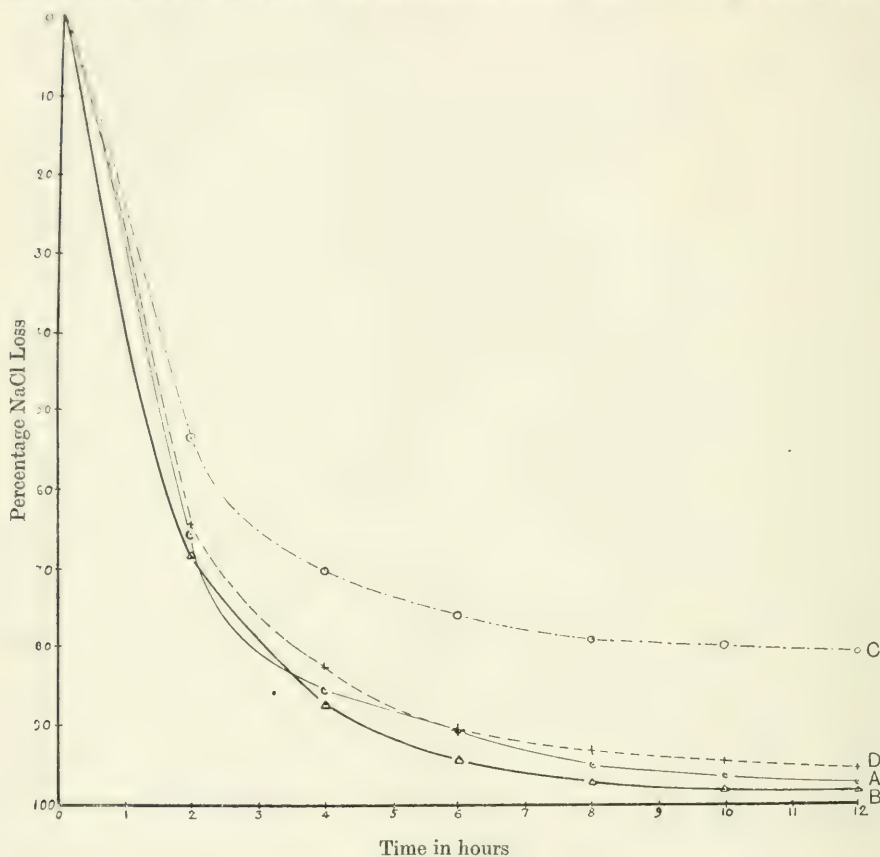


Fig. 4. Diffusion Curves. (Percentage NaCl Loss.)

NaCl Solution	—●—	% NaCl=0.585; A.
Serum Globulin (acid solution)	- - - - -○-	" =0.470; C.
" " (alkaline solution)	- - - - -+	" =0.528; D.
Dialysed Plasma	—△—	" =0.585; B.

Mellanby [1905] and others have studied the influence of salts on the solution or precipitation of the globulin and have recognised that the globulin forms salt compounds in equilibrium with the salt in solution. Pauli concludes that in the solution compounds of globulin with such a salt as NaCl are formed of the type Na-Globulin-Cl. It has been pointed out that, in cases where the effect of electrolytes in solution on living tissues is altered by the addition

of protein to the solution, the action is probably due to the removal of electrolytes from their sphere of action. All such experiments tend to show that certain proteins at least have a chemical (or physico-chemical) affinity for electrolytes.

In order to investigate the matter further certain experiments were carried out to determine the rate of passage of NaCl from an aqueous solution to one of egg white dialysed to such an extent that the globulin had just separated out. The rates at which the salt entered the solution containing a suspension of globulin were determined at first by following the conductivity changes in the protein mixture and also by estimating by chemical analyses the amount of the salt transported to such a solution compared to the rate of transport to water. As the conductivity changes were exactly of the same order as the chemical but were complicated owing to the suspension in the fluid, reference will only be made to the amounts of salt passing from the same volume of 0.03 normal NaCl in one case to the protein mixture and in the other to conductivity water.

Expt. 4. The experiment was carried out in the following way. Filtered egg white was taken, dialysed to the point of separation of the globulin, and 190 cc. of this mixture containing 2.731 per cent. protein were placed, after warming to 37°, in the upper chamber of the diaphragm dialyser. The lower chamber was filled with 300 cc. 0.03 normal NaCl. After a period of four hours had elapsed the fall in NaCl content in the lower fluid was determined.

The total chloride in the lower fluid at the outset was 0.5512 g. in 310 cc. and at the end of four hours, it had fallen to 0.4894, *i.e.* 61.8 mg. NaCl passed to the protein mixture in four hours or 15.45 mg. per hour. Using the same membrane and under the same conditions, but placing conductivity water in the upper chamber, the amount of chloride passing out towards water was found in the experiment to amount to 43.4 mg. in four hours or 10.85 mg. per hour.

The experiment was repeated with a membrane of slightly different permeability, all the other conditions remaining the same, when it was found that 76.27 mg. NaCl passed out to protein in four hours or 19.0 mg. per hour, while to water 52.65 mg. NaCl passed out in four hours or 13.16 mg. per hour. The rate therefore of saline diffusion in the first case to protein and to water respectively was as 15.45 : 10.85 or as 1.42 : 1, and in the second as 19.06 : 13.16 or as 1.44 : 1.

Expt. 5. Using the same membrane that was employed in the last case, the rate of passage of NaCl to dialysed egg white containing 6.08 per cent. protein was tested under exactly the same conditions. It was found that 107.6 mg. NaCl passed out in four hours or 26.9 mg. per hour. The rate therefore in this case with the higher protein content compared to the rate of passage into water was as 26.9 : 13.16 or 2.04 : 1.

Expt. 6. In the next experiment using a fresh diaphragm, the rate of passage of NaCl from a solution of very low concentration, 0.003 normal, to

an egg-globulin suspension also of low concentration (0.14 per cent. protein) was determined. In this case only 10 mg. NaCl passed through in four hours and in the comparable experiment with the same membrane but with conductivity water in the upper chamber 7 mg. NaCl passed through in the same period, the ratio of the rates therefore being 1.42 to 1.

In all the experiments sodium chloride passed more rapidly from the salt solution to the protein mixture (containing a globulin suspension) than to water. It is evident therefore that the decrease in the diffusion rate of sodium chloride from a salt protein solution such as blood plasma during the period of separation of the globulin is intimately related to the increased rate of passage of salt towards a globulin suspension which has as its accompaniment the solution of the globulin. The question as to whether "mechanical" or chemical affinity is the predominating factor in the fixation of NaCl by globulin (in suspension) is difficult to decide.

Expt. 7. With a view to determine whether the solution of the suspended globulin is the governing factor in the process, the proteins in a specimen of dialysed egg white were coagulated by raising the temperature to 80°. The mixture was then cooled to 37° and 230 cc. of the mixture, containing approximately 4.25 per cent. protein, were placed in the upper chamber of the diaphragm dialyser. The lower chamber was filled with 320 cc. 0.03 normal NaCl. The permeability of the collodion diaphragm was such that with the same strength of the salt solution in the lower chamber and conductivity water in the upper, the respective volumes being the same as in the experiment with the heat-coagulated protein mixture, the rate of passage of NaCl to water was 63.6 mg. in four hours or 15.9 mg. per hour.

In the case of the clotted egg white 64.4 mg. NaCl passed from the salt solution to the protein mixture in four hours or 16.1 mg. per hour. The diffusion rates therefore of salt from simple watery solution to water and to clotted egg white were approximately the same.

It appears therefore that the actual solution of the globulin is the determining factor in governing the rate. That the difference in velocity of diffusion is due to variations in the permeability of the membrane owing to blocking up of the pores with consequent diminution of the cross-sectional area of the diffusion path is unlikely, as in all cases when the globulin can undergo solution salt passes more rapidly to the mixture containing the globulin suspension than to water, and in the case of globulin in acid solution the diffusion of NaCl is delayed throughout the whole period although there is only a slight separation of the hydrolysed globulin. The quantitative relationships between the amounts of protein dissolved and the NaCl fixed, as also the influence of variations in reaction on the process, require further study. It is important however to bear in mind that a protein such as globulin forming a large percentage of the colloidal content of blood plasma, tissue fluids, and tissues possesses a certain salt-retaining power which must be of significance in the maintenance of the chloride content of the body.

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XXVIII. THE EFFECT OF METHODS OF EXTRACTION ON THE COMPOSITION OF EXPRESSED APPLE JUICE, AND A DETERMINATION OF THE SAMPLING ERROR OF SUCH JUICES.

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(Received August 11th, 1919.)

A NUMBER of attempts have been made to follow the changes in chemical composition which fruits undergo during ripening and storage. The number of papers dealing with this subject is large, and many of them are published in obscure journals which are difficult of access. The investigations referred to below may however be regarded as typical in respect of methods, and an examination of these methods serves to show the necessity for the adoption of a standard procedure in the preparation of material for analysis, and for a careful estimate of the limits within which the results obtained are significant.

Most of the earlier work has been directed to following the changes of the fruit in respect of its content of sugar and of acid. In some cases acids and sugars have been estimated in a suspension of the pulp [Bigelow, Gore and Howard, 1905]. In others water or alcohol extracts have been made [Kulisch 1892, Browne 1901, Pfeifer 1876, 1877, 1879]. In the majority of cases, however, the juice has been directly expressed, either before or after killing the tissues [Dixon and Atkins, 1913¹]. It is clear that the results obtained by methods so various as these cannot be directly comparable. The value of extraction by water or alcohol evidently depends upon the completeness with which it is carried out, and in most of the extractions which have been made by this method, no attempt appears to have been made to determine this point. Davis and Daish [1914] found that it was necessary to extract leaves in a large Soxhlet apparatus for 18—24 hours with boiling alcohol before all the sugars were removed. No corresponding examination appears to have been made in the case of water extracts, but the extraction by cold water of pulp which has been merely cut up or grated must evidently be slow.

There can be no doubt that for most purposes the most satisfactory method of procedure is to press out the sap from pulp which has previously been

¹ See this paper for further references.

frozen. The advantages of this method have been demonstrated by Dixon and Atkins [1913], and though some doubt exists as to certain of their conclusions [Haynes 1919], yet they show clearly that the sap from untreated tissues is more dilute than that from tissues which have previously been exposed to low temperatures, indicating that some or all of the constituents of the sap are held back in some measure by the untreated pulp. It is possible however that tissues which have been frozen, although considerably more permeable than untreated tissues, still hold back some constituents of the juice. Dixon and Atkins made no attempt to ascertain whether this is the case.

The following experiments have therefore been undertaken with the object of determining how far the sap obtained from apples after freezing and pressing is uniform, *i.e.* whether the first runnings from the press are similar in composition to those obtained later, or whether the tissues still hold back certain of the constituents of the sap. Further, in most of the work hitherto published, the amount of divergence due to sampling error is entirely left out of account; indeed in some cases conclusions have been drawn from the analysis of single fruits. Experiments have therefore been made to ascertain the order of the error due to sampling in investigations of this kind.

A third series of experiments was undertaken with the object of ascertaining with what completeness chemical change in the sap was retarded by cold. It was found more convenient to use an ordinary freezing mixture than to freeze with liquid air, but in the first case the process was necessarily slower, and the apple pulp was usually left in the cold for at least 16 hours.

The following chemical and physical properties were investigated, and some or all were used as a basis of comparison in each case.

(1) Value of P_H .

(2) Value of Δ .

(3) Time of fall in viscometer.

(These were all carried out by ordinary standard methods.)

(4) Electrical conductivity (using a Wheatstone Bridge and a Fitzpatrick commutator).

(5) Acidity (titration), using an iodometric method to avoid difficulties arising from the colour of the juice.

(6) Sugars (reducing sugars and cane sugar by Bertrand's method [1906]; the cane sugar was inverted by 10 per cent. citric acid, as recommended by Davis, Daish and Sawyer [1913]).

METHODS OF EXTRACTION.

The method adopted for extraction was to press out the juice after the tissues had been killed by freezing in an ordinary freezing mixture of ice and salt. A small handpress, worked on the lemon squeezer principle, and provided with a screw, was used. A thin filter cloth was found to be necessary,

and this was saturated with the juice before collection of the sample. The apples were peeled, cut up, and frozen in stoppered jars, and were usually left in the cold for about 16 hours. To ensure uniformity, the frozen pulp was thoroughly mixed before pressing. It was found that the sap obtained by this method from similar samples of pulp gave closely concordant results.

The following points were investigated:

I. *Whether rapid freezing by the use of liquid air produced any alteration in the character of the sample.* It is possible, as has already been stated, that chemical changes may occur during the somewhat prolonged process of freezing in an ordinary freezing mixture. It is also possible that further disruption of the cell membrane may take place when liquid air is used, and that this may affect the character of the sap. For the purpose of comparison, a uniform sample of pulp was divided into two portions, one of which was immediately frozen in liquid air, and pressed out as soon as it had warmed up to the temperature of the laboratory. The other portion was left overnight in a freezing mixture, and then treated in the same way. The chemical and physical properties of the two samples of sap thus obtained were compared.

Table I. *Comparative tests on freezing with liquid air and with a freezing mixture of ice and salt.*

	Liquid air	Freezing mixture
Freezing point depression (Δ)	1.260° C.	1.253° C.
Titration. 10 cc. juice = $N/10$ $\text{Na}_2\text{S}_2\text{O}_3$	9.15 } 9.20 cc. 9.25 }	9.25 } 9.27 cc. 9.30 }
Conductivity. $c \times 10^5$	245	229
Time of fall in viscometer	41.2''	41.2''
Total sugar (after inversion)	8.63 %	8.57 %

It will be seen that the differences obtained by using liquid air instead of a freezing mixture are very small. It is only in the case of electrical conductivity that the difference rises above the limits of experimental error. The reason for this difference has not at present been investigated.

II. *Whether the tissues after freezing are freely permeable to all those constituents of the cell sap present in the expressed juice.* For this purpose the juice was pressed out in several fractions, and these were examined separately. The results are shown in Table II.

In this case again the greater number of the results obtained were uniform. The concentration of acids and of sugars was found to be the same in the first fraction of juice from the press as in the last. The viscosity however varied greatly, indicating that the colloidal constituents of the sap are held back by the tissues. A corresponding difference was also observed in the amount of the precipitate obtained from the two fractions by the addition of alcohol. This precipitate is mainly pectin, which is the principal colloidal constituent of apple-juice.

Table II. *Results of fractional pressing.*

	Fraction	P _H	Δ °C.	Time of fall (viscometer)	Reducing sugars %	Total sugars %
Series I (unripe)	1	3.83	1.44	5' 24"	—	—
	2	3.84	1.42	0' 55"	—	—
	1	3.84	1.43	3' 34"	10.10	11.40
	2	3.85	1.42	1' 35"	9.97	11.35
Series II (ripe)	1	3.87	1.60	3' 43"	10.9	11.75
	2	3.82	1.60	1' 59"	11.05	11.65

	Fraction	Time of fall (viscometer)
Series III (unripe)	1	4' 17"
	2	0' 54"
	1	5' 29"
	2	2' 59"
	3	1' 20"

	Fraction	Δ °C.	Titration	Conductivity (c × 10 ³)	Reducing sugars	Total sugars
Series IV (cold store)	1	1.110	11.3	241	6.20	6.99
	3	1.112	11.15	238	6.25	7.08

Weight of alcohol-precipitate
from 10 cc. juice

1	0.0292 g.
3	0.0090 g.

	Fraction	Δ °C.	Titration	Conductivity (c × 10 ³)
Series V (ripe)	1	1.377	10.7	237
	3	1.380	10.7	239

III. *The probable error due to individual variability in the apples used.*
This was estimated in the usual way by the comparison of a number of samples of sap obtained from individual apples.

The apples on which these experiments were carried out had been kept in cold store for some months, and it is possible that they varied among themselves more than would have been the case had they been more recently picked. Several sets of experiments were made for this purpose. As the results obtained were very similar, it has been thought sufficient to give one set of results, the probable errors being calculated from ten observations¹.

These have been calculated for the purpose of attaining a definite standard of accuracy defined as the probability that the error of any observation may lie within certain limits. This is determined by the number of samples taken.

The standard probability will be taken here as 0.957 (22 to 1), *i.e.* three times the probable error, and the limit below which differences cease to be significant as 5 per cent.

¹ A non-mathematical treatment of the use of probable errors applied to biological work is given by Wood [1911].

Table III. *Probable error attaching to various measurements.*

No. of sample	Freezing point depression (Δ) °C.	Titration	Conductivity ($c \times 10^5$)
1	1.265	9.85	223
2	1.173	9.65	223
3	1.143	12.7	287
4	1.245	10.05	233
5	1.150	10.55	250
6	1.290	8.3	240
7	1.323	10.3	217
8	1.243	10.45	234
9	1.225	10.95	252
10	1.238	9.2	208
Mean = 1.230 ± 0.0127		Mean = 10.20 ± 0.25	Mean = 236.4 ± 4.8

The probable errors of a single observation are:

	$\frac{\text{o}}{\text{o}}$
Δ	0.0412 = 3.2
Titration	0.78 = 7.7
Conductivity	15.2 = 6.4

Since the probable error of the difference of the observations is equal to $\sqrt{2}p$, where p is the probable error of either observation, the above conditions are satisfied by the equation

$$\frac{3 \times \sqrt{2}p}{\sqrt{n}} = 5,$$

where n is the number of observations. The number of samples which must be taken in order that there may be a probability of 0.957 that a 5 per cent. difference is significant is therefore given by the equation

$$n = 2 \left(\frac{3p}{5} \right)^2.$$

The following are the values of n obtained from this equation:

For Δ	7.4
For titration	42.6
For conductivity measurements	29.4

The least number of samples required for these three estimations is therefore 8, 43, and 30 respectively.

These are large numbers and it is to be remembered that the standard of probability assumed is somewhat low. It is more usual to require a probability of not less than 0.969 (30 to 1), *i.e.* a difference of 3.2 times the probable error, in which case the necessary number of samples would be 9, 49, and 34 respectively.

The value of the probable error deduced from such a small number as ten samples is, of course, only approximate but it shows clearly how important is the sampling error. The use of more samples would probably give a somewhat larger probable error but the labour involved in working even with ten samples is considerable.

The importance of the sampling error in measurements of this kind does not appear to have been fully realised by previous workers. It is possible

that the apples used in these determinations differed among themselves more than would have been the case had they been subjected to different treatment, and the error would probably have been considerably reduced if all the apples had been obtained from a single tree, since Kulisch [1892] has shown that apples from different trees may exhibit marked and constant differences; but whether this be so or not it is clear that the sampling error may be very large and from the nature of the material must be far from negligible in any experiments of this kind. Where experiments have been made on two or three fruits only, there can therefore be little doubt that the differences observed are not significant unless they are very large.

SUMMARY.

The juice obtained from apples after freezing has similar properties whether the tissue is frozen rapidly by means of liquid air, or slowly by a freezing mixture.

The sugars and acids present in the tissues can be readily pressed out from the frozen and thawed pulp; colloidal substances, however, of which pectin is the most important, are held back to a large extent by the pulp, so that the amount of these substances present in the expressed juice is no measure of the quantity in which they are present in the tissues.

The samples investigated varied considerably, especially in acid content. These large fluctuations cause the probable error to be very large, and the number of samples required to give results significant within 5 per cent. for lowering of freezing point, for titration, and for conductivity, was 8, 43, and 30 respectively, when the standard of probability assumed was 0.957 (*i.e.* a probability of 22 to 1). The neglect of sampling errors in previous estimations of this nature detracts very seriously from the value of the results obtained and renders many of them worthless.

The work described above has been undertaken in connection with investigations of Cold Storage problems which have been carried out for the Food Investigation Board of the Department of Scientific and Industrial Research. We are indebted to Professor V. H. Blackman for suggestions on which this work has been largely based, and also for much help and advice.

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XXIX. A COMPARISON BETWEEN THE PRECIPITATION OF ANTITOXIC SERA BY SODIUM SULPHATE AND BY AMMONIUM SULPHATE.

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(Received August 13th, 1919.)

My previous work on the factors influencing the concentration of sera has led me to the conclusion that the preliminary heat-denaturation so generally advocated prior to the fractional precipitation of the serum proteins by ammonium sulphate and by sodium chloride does not of necessity materially contribute to the refining and concentrating of antitoxic sera. Recent experiments have shown that, with the technique adopted for the routine work, the heat-denaturation merely induces the precipitation of the antitoxin-bearing protein at lower concentrations of the electrolyte than would be required in the unheated plasma; apparently the heating does not affect the nature of the antitoxin-protein combination.

Further corroborative evidence on this point has been furnished by a study of the fractional precipitation of the serum proteins and of the antitoxin from unheated and from heated antidiphtheritic plasma by sodium sulphate¹.

Several series of experiments were undertaken with samples of oxalated plasma from individual horses and from batches of pooled plasma from different horses. In all cases the plasma was adjusted, either by bringing the [H] to definite values, or by the addition of cresylic acid in stated amounts to the plasma. The method of procedure for each of the series of experiments was as follows: equal volumes of the adjusted plasma were measured into stoppered bottles each of which contained the amount of anhydrous sodium sulphate necessary to bring the proportion of the latter in solution in the plasma to values ranging from 4 to 34 per cent. Owing to the slight solubility

¹ Some years ago Dr C. J. Martin (unpublished observations), worked out a method for the concentration of antitoxic sera by the fractional precipitation of the serum proteins with sodium sulphate rather than with the more toxic ammonium sulphate. Taking advantage of the slight solubility of sodium sulphate at 0° he was able to freeze out the excess of this salt from the Second Fraction precipitates thereby dispensing with the lengthy process of dialysis. For some reason the end products sometimes showed too high a viscosity. The factors underlying this variability were not fully investigated as, in the meantime, Gibson and Banzhaf published their methods involving the heat-denaturation of the proteins.

of anhydrous sodium sulphate in water¹ the respective liquids were kept at 35—40° and constantly stirred until the solution of the sulphate had been effected. Samples were then taken from the bottles and filtered.

The filtration of the hot liquids containing percentages of sodium sulphate in solution greater than those required for saturation at room temperature should be conducted in funnels surrounded with jackets at a temperature of from 30—35°. However, owing to the tendency of solutions of sodium sulphate to remain supersaturated, the filtration of plasma containing 18 per cent., or less, of the anhydrous sulphate could be conducted at the ordinary spring and summer temperature of our London laboratories (about 17°) without the crystallisation of $\text{Na}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ from the mixtures or from the filtrates. It was always advisable to filter large quantities of the plasma containing the higher concentrations of the sulphate through funnels jacketed at 30—35°. The bottles containing the sulphated plasmas were heated in a water bath at 57·5—58° for five to six hours and the hot liquids were filtered, the filtering funnels being jacketed at 35—40° when necessary.

The antitoxin and the protein contents of the filtrates from these heat-denaturated sulphated plasmas were estimated in the usual way, and, after the necessary volume corrections, the data thereby obtained were expressed in the form of curves which were compared with those obtained from similar estimations of the filtrates of the unheated liquids and also with those from the precipitation of samples of the same plasmas by ammonium sulphate.

In accordance with the recent request for the curtailment of the subject matter of communications, curves expressing the experimental data from only three separate batches of antidiphtheritic plasmas are included in this paper.

I. THE PRECIPITATION OF THE SERUM PROTEINS BY SODIUM SULPHATE AND BY AMMONIUM SULPHATE.

Gravimetric estimations of the percentage composition of the respective plasmas were made as regards total protein, pseudoglobulin and albumin with the following results.

	A	B	C
Total protein	7·92 %	8·90 %	7·60 %
Pseudoglobulin	3·18	3·52	2·74
Albumin	2·75	2·68	2·13
Euglobulin, fibrin (by difference)	2·00	2·68	2·73

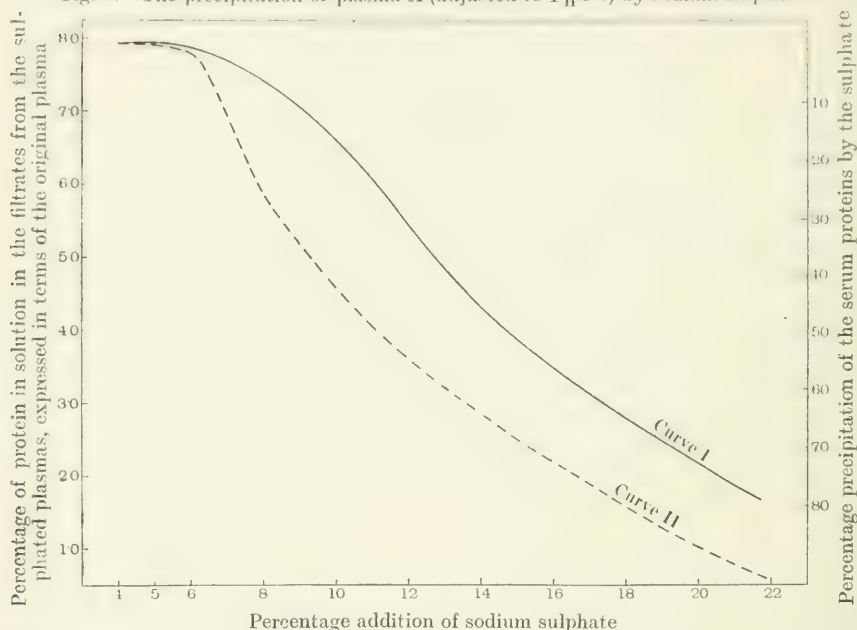
¹ 100 parts of water at t° dissolve a parts of anhydrous Na_2SO_4 :

t°	a parts Na_2SO_4
0	5
13	11·8
16	14·5
20	19·5
30	43

The variations in the proportionate composition of the proteins of the respective plasmas can doubtless be ascribed to variations in the stage of immunisation or of resting at which the horses were bled.

The data obtained for the sodium sulphate precipitation of the plasma A (P_H 8.3) and of the cresylised plasmas A and B, and for the ammonium sulphate precipitation of the plasma A (P_H 8.8) and of the cresylised plasma B, have been represented in the curves in Figs. 1, 2, and 4, and 3 and 5 respectively. In each figure the continuous and the intermittent curves represent the data for the unheated and the heated sulphated plasmas respectively.

Fig. 1. The precipitation of plasma A (adjusted to P_H 8.3) by sodium sulphate



The continuous Curve I ————— represents the precipitation of the plasma A adjusted to P_H 8.3

The intermittent Curve II - - - represents the precipitation of the plasma A adjusted to P_H 8.3 and subsequently heated to 57.5°C. for 5 hours

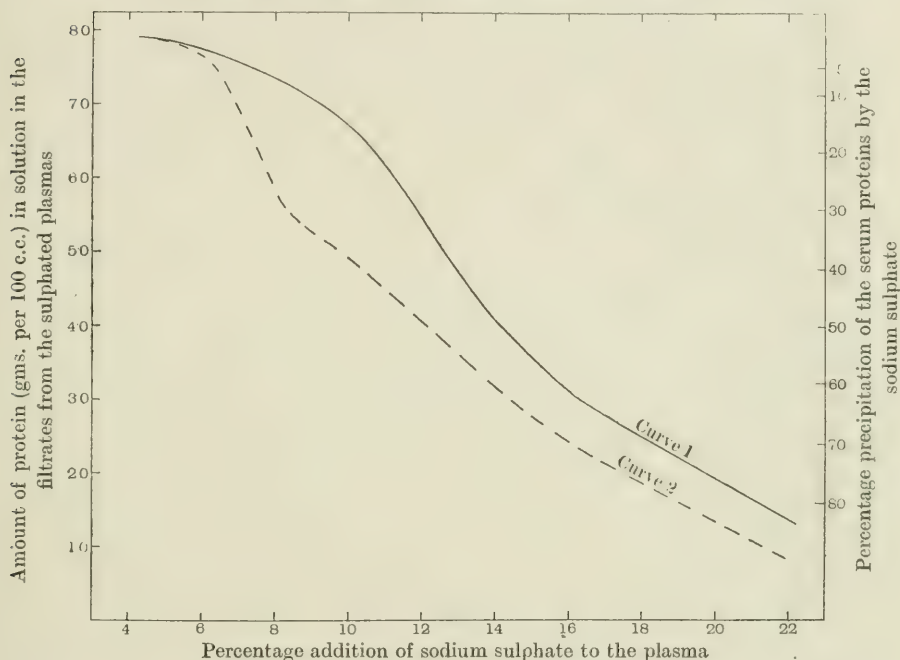
From the relative positions of the points on the curves can be deduced (a) the amount of protein remaining in every 100 c.c. of the filtrates from the sulphated plasmas, and (b) the percentage precipitation of the serum proteins at each stage with sodium sulphate.

It will be seen that the gradients of the curves for the precipitation of the serum proteins from unheated plasma by sodium sulphate are similar to those for the precipitation of the same plasma by ammonium sulphate; with neither salt are there critical points for the precipitation of eu- or pseudo-globulin or of the serum albumin.

It was found that at all stages in the precipitation of the heated plasmas with sodium sulphate there was a marked increase in the precipitability of

the serum proteins similar to that shown by the precipitation of the same plasmas with ammonium sulphate. Moreover, the increased precipitability of the pseudoglobulin by sodium sulphate, registered by the relative shift in the position of corresponding points on the pairs of curves in Figs. 1, 2, and 4 was regulated by the reaction of the plasma, by the temperature at which the plasma was heated and by the duration of the heating at the chosen temperature.

Fig. 2. Showing the precipitation of the serum proteins from plasma A, containing 0.30 per cent. of cresylic acid, by sodium sulphate



The continuous Curve 1 ————— represents the precipitation of the unheated cresylised plasma A

The intermittent Curve 2 — — — — represents the precipitation of the same cresylised plasma after it had been heated to 57.5° C. for 5 hours

On the other hand, in the euglobulin-pseudoglobulin zone the extent of the shift of corresponding points on the curves for the precipitation of the respective heated and unheated plasmas by the two sulphates is greater than that shown in the pseudoglobulin area and than that shown by the data from the precipitation of similarly adjusted solutions of pseudoglobulin [Homer 1919].

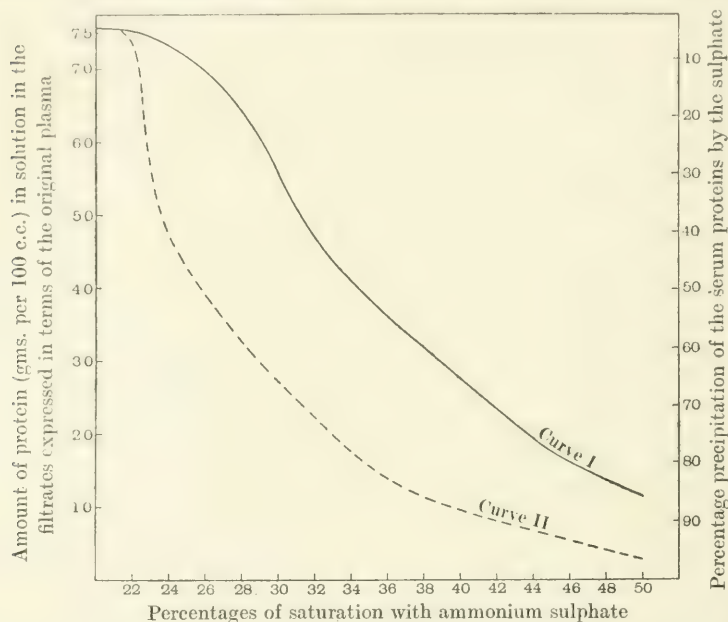
No doubt this differentiation is due to differences in the relative susceptibility of the individual serum proteins to heat-denaturation.

As was to be expected from the general behaviour of colloids the precipitation of the individual proteins from plasma began at and was completed at lower concentrations of ammonium or of sodium sulphate than was

required for their precipitation from their respective separate solutions in saline; there was also a more marked overlapping of the precipitation limits for the individual proteins than was found for their separate solutions.

Thus, from solution in normal saline, the bulk of the pseudoglobulin is precipitated between the limits of 32 and 50 per cent. of saturation with ammonium sulphate, and between 13 and 23 per cent. of anhydrous sodium sulphate; the albumin under similar conditions is mainly precipitated between 54 and 76 per cent. of saturation with ammonium sulphate and between 20 and 34 per cent. of sodium sulphate.

Fig. 3. The precipitation of plasma A (adjusted to P_H 8.8) by ammonium sulphate

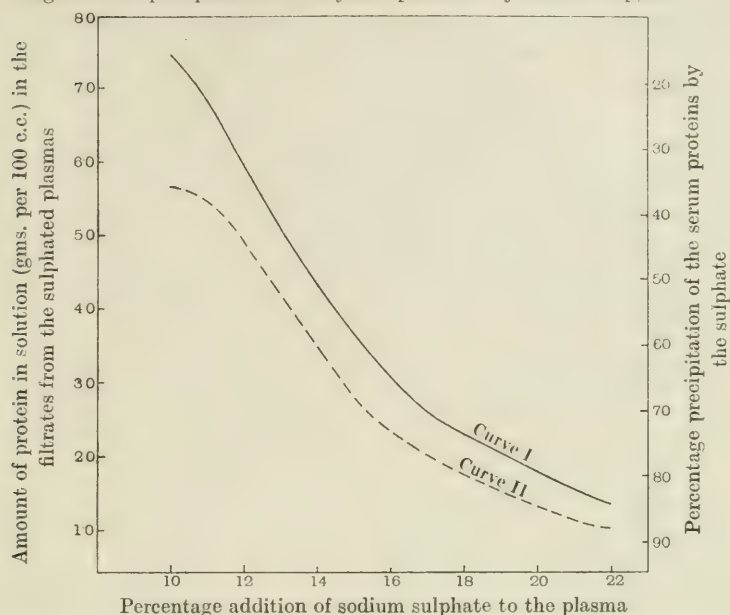


The continuous Curve I ————— represents the precipitation of the unheated plasma A (P_H 8.8)

The intermittent Curve II - - - represents the precipitation of the plasma A (P_H 8.8) after it had been heated to 57.5° C. for 5 hours

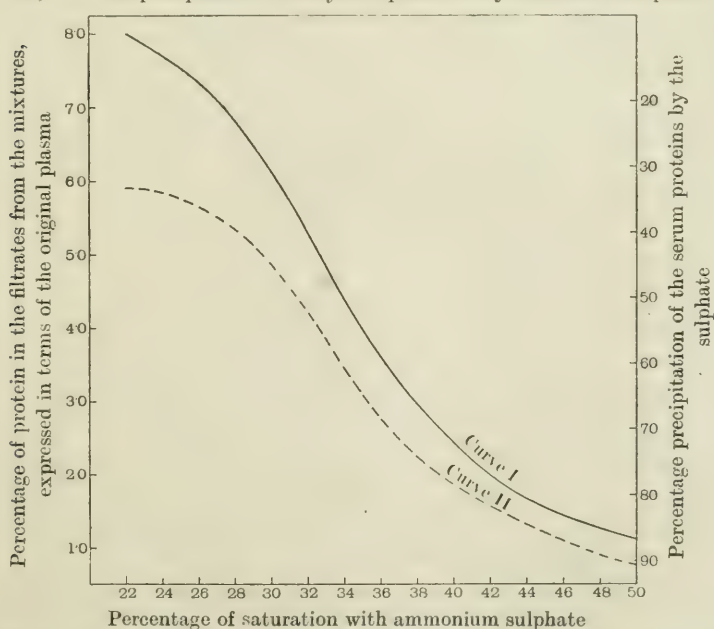
On the other hand there was an appreciable precipitation of pseudoglobulin from unheated unadjusted plasma (P_H 7.4) on the addition of ammonium sulphate to the extent of 28 per cent. of saturation or of 11.5 per cent. of anhydrous sodium sulphate. Moreover, while the precipitation of the pseudoglobulin from the plasma was complete at 46 per cent. of saturation with ammonium sulphate or on the addition of 18.5 per cent. of sodium sulphate, there was, even at these concentrations of the sulphates, an appreciable precipitation of albumin. The precipitation of the latter from the plasma was complete at 67.5 per cent. of saturation with ammonium sulphate or at a concentration of 32 per cent. of anhydrous sodium sulphate.

Fig. 4. The precipitation of cresylised plasma B by sodium sulphate



The continuous Curve I ————— represents the precipitation of the unheated plasma
 The intermittent Curve II - - - - - represents the precipitation of plasma after it had been heated at 57.5° C. for 5 hours

Fig. 5. The precipitation of cresylised plasma B by ammonium sulphate

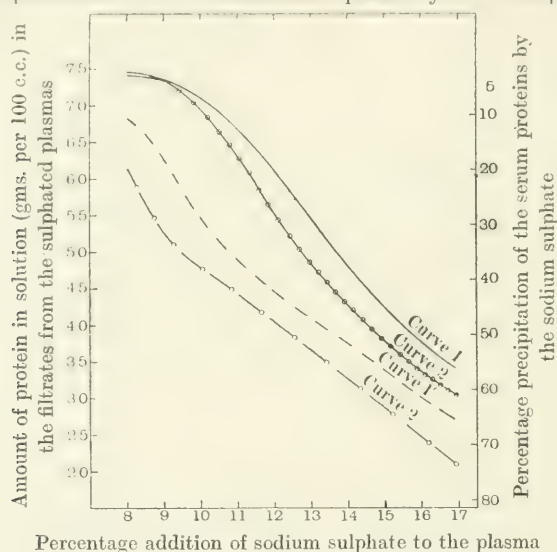


The continuous Curve I ————— represents the precipitation of unheated cresylised plasma B
 The intermittent Curve II - - - - - represents the precipitation of cresylised plasma B after being heated at 57.5° C. for 5 hours

For some time it has been realised that the denaturation of the serum proteins considerably affects their precipitability and that the extent of the overlapping of their precipitation limits from heat-denaturated plasma depends largely on their respective susceptibilities to heat-denaturation.

Recent work has shown that, even with unheated plasma, there are several factors affecting the extent to which the various serum proteins are precipitated by the sulphates under discussion. In this connexion reference may be made to the results which have accrued from preliminary experiments as regards the influence of dilution, of reaction and of the addition of substances such as phenol and cresylic acid to plasma prior to its precipitation by electrolytes.

Fig. 6. Showing the influence of cresylic acid on the precipitability of the serum proteins from plasma and from heat-denaturated plasma by sodium sulphate



The continuous Curve 1 ———— represents the precipitation of protein from plasma C (P_H 7.3)

The continuous Curve 2 —○—○—○ represents the precipitation of protein from plasma C containing 0.30 per cent. of cresylic acid

The intermittent Curve 1' — — — represents the precipitation of protein from plasma C (P_H 7.3) heated at 57.5° C. for 5 hours

The intermittent Curve 2' —○—○—○ represents the precipitation of protein from cresylised plasma C heated at 57.5° C. for 5 hours

It has been found that the limits stated above for the precipitation of the serum proteins were consistent for all the samples of plasma examined in which the protein content varied from 6.5—10 per cent. Dilution of the plasma with water or with normal salt solution did not necessitate a change in the concentration of the precipitating electrolyte until the percentage of protein in solution was reduced to the order of 2.5. At this stage a concentration of 70 per cent. of saturation with ammonium sulphate or of 34 per cent.

of sodium sulphate was required before complete precipitation of the serum protein was effected. The further dilution of the plasma required a further increase in the concentration of the precipitating salt.

The required concentration of the precipitating sulphate was also affected by the reaction of the plasma. However, the variations in the precipitability of the proteins when the reaction of the plasma lay between P_H 5.2 and 9.3 were slight; in more acid or more alkaline plasmas greater changes were detected and, as was to be expected, those shown in plasma more acid than P_H 5.2 were more marked than those found in the alkaline plasma.

The addition of cresylic acid (0.30 per cent.) to unheated and to heat-denaturated plasma produced marked changes in the precipitability of the serum proteins at all stages with the given sulphates. This phenomenon is readily seen from a comparison of the curves in Fig. 6. The curves 1 and 1' respectively represent the precipitation of unheated and of heat-denaturated plasma C by sodium sulphate; the curves 2 and 2' represent the corresponding treatment of plasma C containing 0.30 per cent. of cresylic acid.

The data included in Table I show that the increased precipitability of the serum proteins in cresylised plasma is a function of the amount of cresylic acid present in the plasma.

Table I. *Showing the effect of the addition of varying amounts of cresylic acid on the precipitability of the serum proteins in unheated antidiaphtheritic plasma.*

Percentage addition of cresylic acid to the plasma	Concentration of (A) anhydrous sodium sulphate and (B) ammonium sulphate required for the complete precipitation of the serum proteins from the plasma			
	A		B	
	Na ₂ SO ₄ expressed as		(NH ₄) ₂ SO ₄ expressed as	
	grams per 100 c.c.	gram-molecules per 100 c.c.	Percentage of saturation	gram-molecules per 100 c.c.
0.00	32	0.225	68	0.24
0.15	30	0.21	61.5	0.215
0.30	27	0.19	58.5	0.20
0.45	26	0.18	56	0.19
0.60	25	0.176	54	0.18
0.75	24	0.17	51	0.175

It was also interesting to find that, under the conditions of the experiment, the values for the molecular concentrations of ammonium sulphate required for the complete precipitation of the proteins from the respective plasmas are practically the same as those for sodium sulphate:

The evidence so far obtained in regard to the precipitability of the proteins of unheated and of heat-denaturated plasma indicate that the addition of cresylic acid or of phenol to the extent of 0.5 per cent. or less prior to the prolonged heating of the plasma at 57.5° for five hours does not increase the extent of the heat-denaturation of the serum proteins. The cresylic acid and the phenol materially aid the precipitation of the heat-denaturated protein

which is apt to be so troublesome in the filtration of concentrated antitoxic sera; in this respect their behaviour is analogous to that shown by electrolytes. [Homer 1918, 1.]

In contrast to the experience with non-cresylised plasma, it was found that the dilution of cresylised plasma to a volume four to five times that of its original volume, provided the percentage of cresylic acid in solution were kept constant, did not affect the precipitability of the serum proteins. On the other hand, if, by the dilution, the percentage of cresylic acid in solution were reduced to a value X , then the amount of sodium sulphate or of ammonium sulphate necessary to precipitate the protein was found to be the same as that required by the original undiluted plasma containing X per cent. of cresylic acid.

The above observations on the variations in the precipitability of the proteins of unheated plasma present many points of interest for discussion and elucidation.

Shortly I hope to publish more extensive quantitative data in this connexion.

II. THE ASSOCIATION OF ANTITOXIN WITH THE PROTEINS PRECIPITATED BY THE ADDITION OF AMMONIUM SULPHATE OR OF SODIUM SULPHATE TO ANTIDIPHThERIC PLASMA.

Recent work on the precipitation of solutions of pseudoglobulin by ammonium sulphate [Homer 1919], has demonstrated that there is a comparatively small load of antitoxin attached to the proteins precipitated by ammonium sulphate in the pseudoglobulin-euglobulin zone, that the remainder of the antitoxin is evenly distributed throughout the fractions of the pseudoglobulin precipitated at higher concentrations, and moreover that, at all stages in the latter region, the increased precipitation of proteins due to heat-denaturation is a linear measure of the accompanying increased precipitation of antitoxin.

These results together with my previous observations on the factors influencing the concentration of heat-denaturated plasma by ammonium sulphate have led me to the conclusion that the ratio of antitoxin to protein in the final products can only be increased by a removal of the proteins to which little or no antitoxin is attached, viz. those of the euglobulin-pseudoglobulin or of the pseudoglobulin-albumin zones.

Further evidence in this respect is given by the fractional precipitation of the unheated plasma by ammonium sulphate and by sodium sulphate respectively.

In Table II have been included the data obtained from the fractional precipitation of separate volumes of unheated antidiphtheritic plasma A and of the same plasma to which an addition of 0.30 per cent. of cresylic acid had been made. The method of procedure was the same as that used in the fractionation of pseudoglobulin [Homer 1919].

Table II. *The fractional precipitation of unheated antidiphtheritic plasma by ammonium sulphate*.*

Limits of saturation with ammonium sulphate between which the protein fractions were isolated	Percentage of the total protein appearing in the dialysed fraction	Percentage of the total antitoxin appearing in the dialysed fraction	Degree of concentration found in the end products, calculated, for purposes of comparison, on the basis that they contain 20 % of protein
<i>Non-cresylised Plasma.</i>			
33-50	45.5	77	4.2
36-50	37.0	70	4.95
38-50	23.3	48	5.45
36-44	22.4	60	7.1
38-44	10.5	38	9.5
<i>Cresylised Plasma.</i>			
32-45	40.8	70	3.8
33-45	34.1	68	5.1
34-45	25.8	62	5.4
36-45	21.0	51	5.5
38-45	10.4	28	6.0
34-50	27.3	62	5.1

* The antitoxin precipitated with the various First Fraction precipitates was completely recovered by extraction of the precipitates with a saturated solution of salt.

Table III. *The fractional precipitation of unheated cresylised antidiphtheritic plasma by sodium sulphate*.*

Limits of percentage addition of sodium sulphate to the plasma between which the protein fractions were isolated	Percentage of the total protein appearing in the dialysed fraction	Percentage of the total antitoxin appearing in the dialysed fraction	Degree of concentration found in the end products, calculated, for purposes of comparison, on the basis that they contain 20 % of protein
8-16.5	59.5	97	3.6
10-16.5	53.0	97	4.3
11-16.5	47.0	97	4.6
12-16.5	36.0	80	5.0
13-16.5	26.0	60	5.5
14-16	14.6	40	6.5
10-18	57.0	97	3.7
10-20	63.0	97	3.45

* The antitoxin precipitated with the various First Fraction precipitates was recovered by extraction with a saturated solution of sodium chloride.

In Table III have been embodied the corresponding data obtained from the fractional precipitation of unheated cresylised plasma B by sodium sulphate. The method of procedure was as follows.

To separate volumes of cresylised plasma (0.30 per cent.) was added the amount of anhydrous sodium sulphate necessary to bring the concentration of the latter to the value chosen for the precipitation of the First Fraction,

viz. X per cent. The respective liquids were kept at a temperature of $35-40^{\circ}$ ¹ and were constantly stirred until complete solution of the sulphate had taken place. The hot liquids were then filtered through paper at room temperature. The First Fraction precipitates were washed with a volume of an X per cent. solution of anhydrous sodium sulphate in water equal to that of the plasma taken for fractionation, the washings were filtered and added to the main filtrate. To the measured volume of filtrate and washings was added the weight of anhydrous sodium sulphate necessary to bring the concentration of the latter in solution up to Y per cent.² The liquids were again heated to $35-40^{\circ}$ and kept at this temperature until complete solution of the sulphate had taken place. The hot liquids were then filtered, and as before (*ante*, p. 279), where the concentration of sulphate was 18 per cent. or less, the filtration was conducted at room temperature; with greater concentrations of the sulphate it was necessary to enclose the filtering funnels in water jackets kept at about 35° .

Where the value for Y was not greater than 18 per cent. the protein fractions, thus isolated in the Second Fraction precipitates, were drained and pressed quickly in the usual way. At greater concentrations of the sulphate, owing to the tendency of the sodium sulphate to crystallise out from the fluid adhering to the precipitate, it was necessary to conduct the pressing operations in the hot room.

The pressed precipitates were dialysed and to the residues from dialysis were added 0.35 per cent. of cresylic acid, and 1 per cent. of sodium chloride. The protein and antitoxin content of the end products were respectively estimated and from the results were calculated the degree of concentration and also the percentages of the total protein and of the total antitoxin associated with the various fractions.

The data included in the tables show that, by a suitable selection of the precipitation limits for the isolation of the antitoxin-protein fraction, results can be obtained from unheated plasma similar to those hitherto obtained from heated plasma, provided that the precipitating limits be chosen so as to exclude albumin, euglobulin and the lower fractions of pseudoglobulin to which a relatively small proportion of the antitoxin is attached.

More detailed work was then undertaken to furnish evidence both as to the proportional distribution of the antitoxin throughout the protein fractions precipitated at progressively increasing concentrations of sodium sulphate, and also as to whether the heat-denaturation (at 58°) effects changes in the nature of this association.

Several batches of plasma were treated in the manner described in Section I (p. 279). Estimations of the protein and the antitoxin content of the filtrates

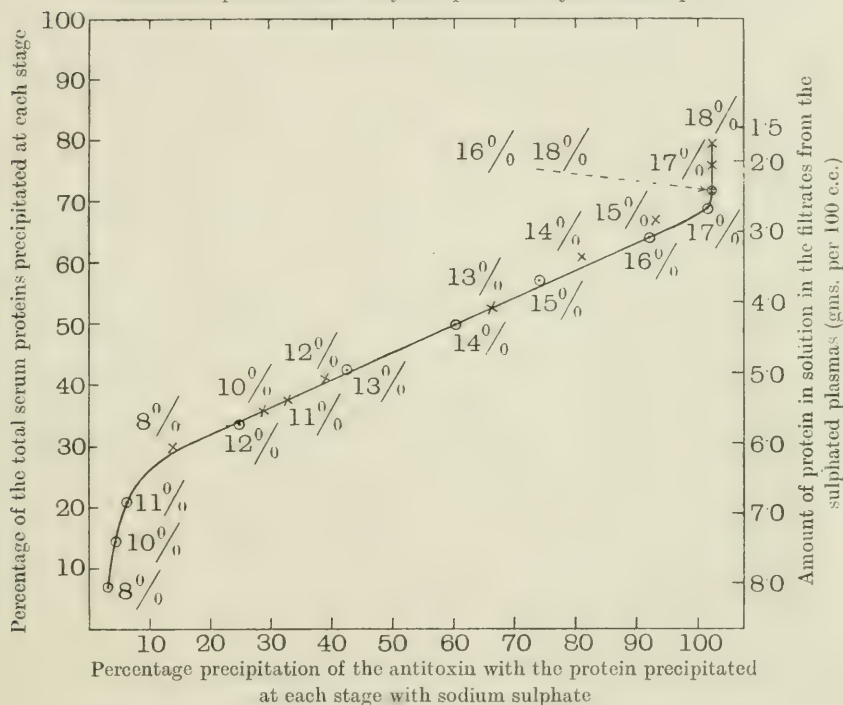
¹ No appreciable heat-denaturation of the serum proteins takes place during the short time required for the solution of the sulphate.

² To every 100 c.c. of liquid add $Y - X$ grams anhydrous sodium sulphate; the volume changes following the solution of the sulphate in the plasma can be neglected.

from the unheated and from the heated sulphated plasmas were made and from the data thus obtained were calculated (a) the amount of protein remaining in every cc. of the filtrates, and (b) the respective precipitations of the serum proteins and of the antitoxin at each stage with the sulphate.

It was found that in the *unheated* plasmas the preliminary treatment of the plasma affected the limits for the precipitation of the antitoxin-bearing proteins. Thus, in plasma of which the reaction lay between $P_H 7.4$ and 8.3 the main part of the antitoxin-bearing proteins was precipitated in the

Fig. 7. Showing the relationship between the precipitation of the antitoxin and of the serum proteins from cresylised plasma B by sodium sulphate



The circumscribed points ○ represent the data for the unheated plasma.

The points at the intersection of two lines × represent the data for the heat-denatured plasma

The figures by the side of each point indicate the concentration of sodium sulphate in the plasma

fraction isolated between 12 and 18.5 per cent. of sodium sulphate: from the same plasma to which 0.30 per cent. of cresylic acid had been added, the precipitation limits were respectively reduced to 11.5 and 16.5 per cent. of the sulphate¹. Furthermore, in the former case the limits were raised further by the dilution of the plasma; in the latter case they were lowered by an increase in the concentration of the cresylic acid in the plasma.

¹ A similar lowering of the precipitation limits was seen in the precipitation of cresylised plasma by ammonium sulphate.

In the *heated* plasmas the precipitation limits with sodium sulphate were considerably lower than those given above for the unheated liquids, and, as was anticipated, the increased precipitability of the antitoxin proved to be a function of the heat-denaturation of the antitoxin-bearing proteins.

The data furnished from the study of the precipitation of the cresylised plasma B by sodium sulphate have been embodied in the curve in Fig. 7 in which the circumscribed points and those at the junction of a cross respectively denote the values obtained from the unheated and the heated liquids.

A study of the curve shows that, in the *unheated* cresylised plasma, only a small amount of antitoxin (less than 5 per cent.) was precipitated in the pseudoglobulin-euglobulin zone, viz. at concentrations from 9—11.5 per cent. Beyond this stage the points representing the relative precipitation of antitoxin and protein lie on a straight line the slope of which remains constant until the whole of the antitoxin has been precipitated.

In the *heated* cresylised plasma there was an increased precipitation of protein and of antitoxin at each of the stages investigated. Even at a concentration of 8 per cent. of sodium sulphate there was an appreciable precipitation of denaturated pseudoglobulin and its associated antitoxin; the precipitation of antitoxin and pseudoglobulin was complete on the addition of 15.5 per cent. of sodium sulphate. The position of the corresponding points on the curve shows that, in heated plasma also, there is a direct proportionality between the precipitation of antitoxin with the protein at each stage with the sulphate, and, furthermore, that the points representing this relationship fall on the same straight line as that connecting the points obtained for the unheated liquids.

The gravimetric estimations of the relative amounts of the individual proteins precipitated at various stages with the sulphate indicated that the precipitation of antitoxin from the plasma coincided with that of the pseudoglobulin.

Calculations were accordingly made of the percentages of the total antitoxin and of the total pseudoglobulin precipitated at each stage with the sulphate and the data thus obtained were incorporated in the curve in Fig. 8, the positions for the data from unheated and heated plasmas being indicated by circumscribed and by crossed points respectively.

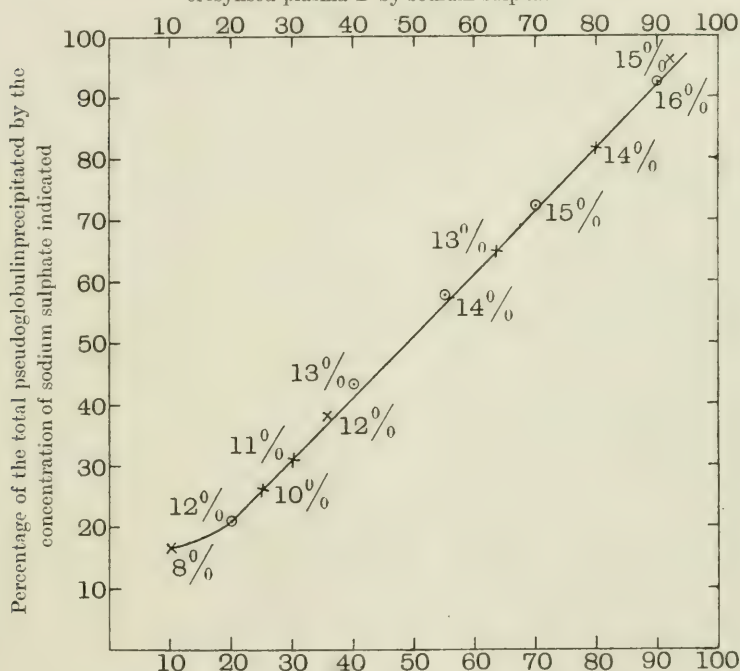
It will be seen that, in the unheated liquids, there is a comparatively small load of antitoxin associated with the fractions of the pseudoglobulin precipitated in the pseudoglobulin-euglobulin zone, and that, beyond this zone *the percentage of the total pseudoglobulin successively precipitated by increasing concentrations of the sulphate is a linear measure of the percentage of the total antitoxin associated with the precipitate.*

In the heated liquids there was no disturbance of this relationship between the relative precipitation of pseudoglobulin and antitoxin. The heat-denaturation merely served to induce the precipitation of a particular pro-

portion of the pseudoglobulin and its associated antitoxin at a concentration of sodium sulphate lower than that required for their precipitation from the unheated plasma.

These results furnish evidence in confirmation of the conclusions drawn from the investigation of the fractional precipitation of the pseudoglobulin by ammonium sulphate, viz. that from the point of view of reducing the proportion of protein associated with the antitoxins of sera, there are no practical advantages to be gained by the preliminary heat-denaturation of the serum proteins [Homer 1919].

Fig. 8. Showing the relative precipitation of antitoxin and pseudoglobulin from cresylised plasma B by sodium sulphate



Percentage of the total antitoxin precipitated with the pseudoglobulin

The points \circ refer to data obtained from the precipitation of unheated plasma

The points \times refer to data obtained from the precipitation of heat-denatured plasma

The figures by the side of each point indicate the concentration of sodium sulphate used for precipitation

III. THE APPLICATION OF THE ABOVE RESULTS TO THE ROUTINE CONCENTRATION OF SERA.

The question as to whether the routine concentration of antitoxic plasma shall be carried out with unheated or with heat-denatured plasma can only be decided after a careful comparison of the respective end products as regards (a) their ease of filtration through Berkfeld and through white Doulton filter candles, and (b) the clinical after-effects of their administration to the animal or to the human.

Unfortunately, through the lack of the necessary training, I cannot produce evidence on the latter point other than that referred to in a previous note [Homer 1918, 2] in which attention is drawn to variations in the toxicity of cresylic acid in plasma according as the serum proteins in the latter have been subjected to heat-denaturation or not.

The conditions regulating the preparation of satisfactory end products from the ammonium sulphate or from the sodium sulphate fractionation of unheated and of heat-denaturated plasma have been studied separately and the following observations have been made.

(i) *The filtration of the end products from the concentration of unheated plasma.*

In order to obtain clear end products it is essential to fix the precipitation limits with ammonium sulphate or with sodium sulphate so as to ensure the elimination of euglobulin. Satisfactory products are obtained from the dialysis of the protein fractions isolated from unheated plasma ($P_H 7.4-8.3$) between concentrations of ammonium sulphate of which the lower limit is not less than 34 per cent. of saturation. In the cases where sodium sulphate is used the lower limit should not be less than 11.5 per cent.

Where the lower limit for the precipitation with ammonium sulphate was 33 per cent. of saturation or less, or was less than 11.5 per cent. with sodium sulphate, the end products were opalescent and filtered badly; the filter candles during the filtration became coated with a slimy deposit of euglobulin which retarded and ultimately stopped the process.

The Second Fraction precipitates isolated from unheated plasma ($P_H 8.3$) between 34 and 46 per cent. of saturation with ammonium sulphate or between concentrations of 12 and 18.5 per cent. of sodium sulphate contained the bulk of the antitoxin. The corresponding precipitates from cresylised plasma (0.30 per cent.) contained a lower proportion of the antitoxin for the reason that the presence of the cresylic acid induced an increased precipitation of the antitoxin-bearing proteins with the First Fraction precipitates.

The antitoxin carried down with the First Fraction whether precipitated by sodium sulphate or by ammonium sulphate, was, in all cases, recovered by extraction of the precipitates with a saturated solution of sodium chloride.

Contrary to the experience with heat-denaturated plasma it was found that the end products from the fractionation of unheated cresylised plasma between the above limits with ammonium sulphate were opalescent and filtered less readily than those obtained from the non-cresylised plasma. Further experiment showed that in the cresylised plasma the concentration of the precipitating sulphate required to ensure the agglutination and filtration of the cresylic acid-protein complex with the First Fraction precipitates was greater than that required for the complete precipitation of euglobulin.

The same phenomena were exhibited to a less marked degree in the precipitation of cresylised plasma by sodium sulphate.

These results indicate that, in the concentration of antitoxic plasma by the fractional precipitation of the unheated plasma, the preliminary addition of cresylic acid is disadvantageous. For, not only does it reduce the proportion of antitoxin associated with the Second Fraction precipitates, but it also leads to the production of less readily filterable end products than are furnished from the similar treatment of non-cresylised plasma.

(ii) *The filtration of the end products from heat-denaturated plasma.*

In previous communications I have described techniques [Homer 1917, 1918, 3] which gave the best results for the fractional precipitation of heat-denaturated plasma by ammonium sulphate. I am still convinced that more consistent and more reliable results are to be obtained from the preliminary adjustment of the reaction than from the alternative procedure of adding 0.30 per cent. of cresylic acid to the plasma.

In the present investigation also it has been found desirable to regulate the heat-denaturation of the serum proteins prior to their fractional precipitation with sodium sulphate.

The directions previously given for the suitable adjustment of the reaction of the plasma may be somewhat simplified. Satisfactory values for the heat-denaturation are obtained by the adjustment of the reaction of the plasma, so that 10 c.c. show a very faint green tinge with α -naphthol-phthalein; the colour change is readily detected when the plasma is viewed slantwise.

A further simplification of the routine technique in regard to the heating processes hitherto advocated in order to ensure the required heat-denaturation and the subsequent separation of heat-denaturated protein with the First Fraction precipitate can be effected as follows. To the adjusted plasma, prior to its being heated, is added the amount of sodium sulphate or ammonium sulphate required for the precipitation of the First Fraction precipitates. The sulphated mixtures are heated to 58° and kept at that temperature for four to five hours. They are then filtered and from the combined volume of the filtrates and washings are precipitated the Second Fraction precipitates in the usual way. By this method of procedure the two stages of the heating process are reduced to one.

However, in the case of the ammonium sulphate precipitates the advantage thus gained by combining the two stages of the heating is minimised by the greater losses of antitoxin subsequently experienced; the latter are no doubt due to the hydrolysis of ammonium sulphate and its dissociation into its component parts during the prolonged heating.

On the other hand, as sodium sulphate is the salt of a strong acid and a strong base, no such secondary complications arise.

Presumably this difference in the nature of the two sulphates also accounts for the somewhat sharper line of demarcation shown in the precipitation of eu- and pseudo-globulin from plasma by sodium sulphate than when

ammonium sulphate is employed; a phenomenon which favours the use of the former sulphate in the concentration of antitoxic sera.

In the heated plasmas containing sodium sulphate, in which the heat-denaturation had been regulated as above, the precipitation of the euglobulin was complete at a concentration of 8 per cent. of anhydrous sodium sulphate, but the separation of the precipitated protein by filtration through paper was impossible. Just as with ammonium sulphate, the indifferent filtration of the First Fraction mixtures was due to the presence of incompletely agglutinated particles of heat-denaturated protein, and unless the separation of the latter was ensured at this stage, the filtration of the end products was impossible. For this purpose it was found necessary to increase the concentration of sodium sulphate to 10 per cent., but, in order to guarantee the rapid filtration of the First Fraction precipitates it was advisable to use 11 per cent. of the sulphate, a procedure which entailed the precipitation of at least 35 per cent. of the antitoxin with the protein eliminated at this stage. However, provided that the heat-denaturation has not been greater than that recommended for the routine work, the antitoxin thus precipitated with the First Fraction precipitates can always be recovered by the extraction of the precipitate with a saturated solution of salt.

Those engaged in the routine work must be prepared to find that the precipitation limits discussed above may not give quite the same results when applied to the concentration processes carried out in their laboratories. There are many conditions which affect the precipitability of the serum proteins, and in the routine work the important factor, dilution, is apt to be forgotten. Thus, after the removal of the First Fraction, the solution contains considerably less protein than the original plasma; the protein content is further reduced by the addition of the filtered washings, a procedure which tends to modify the precipitation limits for the proteins (*ante*, p. 284). The investigation of practical details of this nature are best carried out by the individual worker under the conditions which prevail in his particular laboratory.

SUMMARY.

The results of the investigation have shown that:

(1) In the precipitation of the serum proteins by sodium sulphate there are no critical points marking the limits for the precipitation of the individual proteins.

(2) The concentration of either ammonium sulphate or sodium sulphate required for the precipitation of the serum proteins is affected by the reaction and by the dilution of the plasma and by the addition of cresylic acid to the plasma. In the latter case the extent of the increase is a function of the concentration of cresylic acid in the plasma.

(3) Within the limits of the error of the experiment, the respective decreases in the molecular concentration of sodium sulphate and of am-

monium sulphate measured under the conditions given in (2) are practically identical.

(4) The percentage precipitation of the antitoxin with the proteins precipitated at various concentrations of sodium sulphate is a linear measure of the percentage precipitation of the antitoxin-bearing proteins. This relationship is undisturbed by the heat-denaturation of the serum proteins induced during the heating of adjusted plasma at 58° for four to five hours.

(5) In the concentration of antitoxic plasma by its fractional precipitation with sodium sulphate or ammonium sulphate, results can be obtained by the suitable fractionation of the unheated plasma similar to those which have hitherto been obtained with heated plasma.

(6) While the addition of cresylic acid materially aids the concentration of heat-denaturated plasma, its use with unheated plasma is to be deprecated.

(7) The agglutination of the particles of precipitated protein in the eu- and pseudo-globulin zone seems to be more satisfactory with sodium sulphate than with ammonium sulphate.

(8) As sodium sulphate, in contradistinction to ammonium sulphate, does not hydrolyse in solution, the sodium-sulphate-plasma First Fraction mixtures can be heated for four to five hours at 58° without loss of antitoxin. This method of procedure favours the production of clearer end products than would result from conducting the heating in the two stages adopted in the ammonium sulphate method.

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XXX. THE DIRECT REPLACEMENT OF GLYCEROL IN FATS BY HIGHER POLYHYDRIC ALCOHOLS. PART I. INTERACTION OF OLEIN AND STEARIN WITH MANNITOL.

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(Received August 18th, 1919.)

GLYCEROL is required in abnormally large quantities during war and therefore just at such times as a shortage may be expected of oils and fats which are the essential raw materials in the manufacture of glycerol as at present practised. As the by-products in the manufacture have but a limited application as food-stuffs, various suggestions have been made with a view to extending this application and, among others, that of converting the fatty acids into ethyl and other esters has been considered. It is but a short step from this to the idea of esterifying the acids with polyhydric alcohols similar in type to glycerol and this again suggests the possibility of using sugars and their simpler derivatives for a like purpose. The latter line of inquiry appears especially promising inasmuch as many of the substances in this group are themselves valuable as food-stuffs.

We have been associated with Professor R. Robinson in considering the chemical aspects of such problems and in consultation with him decided to attempt a more direct solution than esterification of the preformed fatty acids.

It is well known that small quantities of acids or alkalis facilitate the exchange of alcohol residues in esters and accelerate the attainment of equilibrium in the resulting system. In those instances in which one of the products can be removed from the sphere of action a complete conversion of one ester into another may be effected.

It was anticipated that by bringing together, in presence of an acid or alkaline catalyst, a natural oil or fat and a polyhydric alcohol having a higher boiling point than glycerol the latter might be removed by distillation under reduced pressure.

Our first experiments were made with mannitol as the polyhydric alcohol and the catalyst used was sodium ethoxide, the alcohol arising therefrom being carried over with the glycerol formed during the reaction. The results justified our anticipations and although the products are not simple esters

of mannitol itself as we at first supposed, they resemble true glycerol fats in appearance and to some extent in taste and are reported by Professor W. D. Halliburton and Dr Drummond [1919] to be assimilated by rats to much the same extent as is olive oil.

Interaction of olive oil with mannitol. Formation of "Mannitol olive oil."

The olive oil used was a good commercial sample and had an acid value corresponding with 2.5 per cent. of free oleic acid.

100 g. of this oil was mixed in a Claisen distillation flask with 31 g. of pure mannitol and 1.5—2 per cent. of sodium ethoxide (freshly prepared by evaporating a solution of sodium in alcohol and heating gently until the mass appeared dry). One thermometer was kept with its bulb immersed in the mixture itself (the temperatures recorded by this are indicated hereafter by the letter T) and another with its bulb immersed in the issuing vapours in the exit tube of the flask (temperatures indicated by the letter t).

Experience having shown that on the small scale no harm resulted therefrom, the heating was usually carried out for convenience by direct contact with the flame of a Bunsen burner. Alcohol in small quantities appeared to be evolved first ($T = 87-90^\circ$, $t = 25-30^\circ$); later the mannitol crystals melted and two fluid layers were formed in the flask ($T = 140^\circ$) while considerable frothing ensued which continued as T rose to 215° . Here frothing subsided to some extent and as the heating went on the two fluid layers began to react, the upper one becoming turbid, and appearing to boil. Water, contaminated with a little oily or solid matter, distilled at this point ($t = 54^\circ$) and continued to do so as the temperature rose. A steady bubbling now took place (T steady at $236-240^\circ$ during 10—15 minutes; t about 52°), and the lower layer decreased in bulk. With further heating a thick fluid began to distil (t rose to 170° while T was raised to 270°) and the two layers in the flask merged into one. The heating was not pressed much further as some darkening in the product occurred if T was allowed to rise to 275° and very little more distillate was obtained.

Examination of distillate. This formed a thick viscid fluid on which floated a little oil; it was diluted with water, extracted with ether to free it from oily impurities and the aqueous solution was evaporated on the steam bath. A thick, viscid, sweet liquid remained (10 g.) which on distillation yielded 8 g. of a thick, almost colourless fluid, boiling at 179° at 14 mm. pressure. This was practically pure glycerol and was identified by conversion into the crystalline benzoate. As the amount of glycerol theoretically obtainable from the quantity of olive oil used is 10.4 g. it is evident that the yield was excellent in spite of losses inevitable in the manipulation of such comparatively small quantities of material.

Examination of residue. The fluid remaining in the flask was dissolved in ether and shaken with water, the whole being treated drop by drop with

acetic acid until a permanent slight acidity towards litmus was attained. The ethereal solution was separated and shaken several times with water to remove sodium salts together with any glycerol and mannitol or its anhydrides which might be present, though in point of fact when the foregoing procedure was followed the aqueous fluids yielded only very small quantities of residue, mainly sodium acetate, so that practically no free glycerol or mannitol was present.

After a preliminary drying over sodium sulphate and evaporation of the ether, the residual oil was more fully dried by heating it in a vacuum at 200—220° for several hours while a continuous stream of air was passed through it.

The product is an oil with a brownish yellow colour and a very faint odour. In taste and fluidity it is not dissimilar to olive oil. When kept for some weeks it gradually becomes somewhat cloudy and this effect appears to be due to deposition of a fine powder (stearates?) which may be removed by filtration. Professor Halliburton kindly undertook the examination of the properties of this oil when used as a food and a report on the subject appears elsewhere in this *Journal* [Halliburton and Drummond, 1919].

We have formed definite views on the chemical character of the oil only after consideration not only of its percentage composition and the amount of free and combined acid, but also of free hydroxyl, which was determined by means of magnesium methyl iodide in amyl ether.

By the interaction of mannitol and glyceryl oleate there may be formed a number of oleates in which from one to six of the hydroxyls of mannitol are esterified, as well as a series of wholly or partially esterified derivatives of mannitan and of isomannide, compounds which are derived from mannitol by loss of one and two molecules of water respectively. The percentages of carbon and hydrogen in many of these esters hardly admit of their being used to distinguish the esters from one another in a mixture, even if the entire absence of any esters of glycerol could be regarded as quite certain. On the other hand the percentages of combined acid, calculated as oleic acid, and especially of the free hydroxyl in the several possible esters vary very considerably as is shown in the following table from which some esters have been omitted because their composition differs so widely from that of the "mannitol olive oil" that they could only be present there if at all in quite small proportions.

While it seems possible that several or all of the above esters are present to some small extent in "mannitol olive oil" a careful examination of the table will show that in every instance but two there is at least one characteristic figure for each which differs widely from the one found experimentally. The two exceptions are the di-oleates of mannitan and isomannide.

Compositions of various esters of Oleic Acid.

Compound	Mol. wt. and formula	% OH	% combined acid	% C	% H
(1) Mannitol penta-oleate	$C_{98}H_{174}O_{11}$ (1502)	1.13	93.8	76.69	11.58
(2) Mannitol tetra-oleate	$C_{78}H_{142}O_{10}$ (1238)	2.7	91.1	75.6	11.47
(3) Mannitol tri-oleate	$C_{60}H_{110}O_9$ (974)	5.22	86.85	73.92	11.29
(4) Mannitol di-oleate	$C_{42}H_{78}O_8$ (710)	9.57	79.43	70.98	10.98
(5) Mannitan tri-oleate	$C_{60}H_{108}O_8$ (956)	1.77	88.49	75.31	11.29
(6) Mannitan di-oleate	$C_{42}H_{76}O_7$ (692)	4.91	81.5	72.83	10.98
(7) Isomannide di-oleate	$C_{42}H_{74}O_6$ (674)	0	83.68	74.77	10.97
(8) Isomannide mono-oleate	$C_{24}H_{42}O_5$ (410)	4.01	68.78	70.24	10.24
Olive oil	$C_{37}H_{101}O_6$ (884)	0	95.7	77.36	11.76
Found in "mannitol olive oil"	—	2.17	83.65	72.82	10.77

Interaction of mannitol with stearin. Formation of "Mannitol stearin."

Mannitol and stearin were mixed and heated in a vacuum with a little sodium ethoxide under much the same conditions as in the experiments where olive oil instead of stearin was used. The non-volatile residue was dissolved in chloroform, in which it dissolved more freely than in ether, and the resulting solution was treated with acetic acid and water as described in the preceding experiments. The washed and dried product was solid at ordinary temperatures. It was easily soluble in cold chloroform, hot petroleum (B.P. 80—100°), hot ethyl acetate, acetone or benzene. It dissolved in hot alcohol but was nearly insoluble in the cold. It separated from acetone on cooling as a granular powder melting at 67—72°.

Advantage was taken of the solid character of this product to do what was not possible in the case of the material from olive oil, namely to separate it into two main fractions by crystallisation from acetone. It was anticipated that if there were present any appreciable quantities of impurities markedly different in character from the main mass, evidence of this would be obtained by analysis of different fractions. In point of fact however no appreciable difference was found.

It is also interesting to observe that if the molecular proportion of mannitol to stearin used in the original reaction is increased from 1.5 to 2.25, the product is not appreciably altered in percentage composition and it seems probable that any excess of mannitol passes over with the glycerol during the heating with stearin in the first process or is washed out afterwards.

In the following table are exhibited the four characteristic data for the series of stearic esters derived from mannitol and its dehydration products.

Compositions of various esters of Stearic Acid.

Compound	Mol. wt. and formula	% OH	% combined acid	% C	% H
(1) Mannitol penta-stearate	$C_{96}H_{183}O_{11}$ (1512)	1.12	93.91	76.19	12.16
(2) Mannitol tetra-stearate	$C_{78}H_{150}O_{10}$ (1246)	2.72	91.1	75.12	12.03
(3) Mannitol tri-stearate	$C_{60}H_{116}O_9$ (980)	5.2	86.93	73.47	11.83
(4) Mannitol di-stearate	$C_{42}H_{82}O_8$ (714)	9.52	79.55	70.58	11.48
(5) Mannitan tri-stearate	$C_{60}H_{111}O_8$ (962)	1.76	88.56	74.83	11.85
(6) Mannitan di-stearate	$C_{42}H_{80}O_7$ (696)	4.88	81.6	72.41	11.49
(7) Isomannide di-stearate	$C_{42}H_{78}O_6$ (678)	0	83.9	74.32	11.50
(8) Isomannide mono-stearate	$C_{24}H_{41}O_5$ (412)	4.12	68.93	69.9	10.67
Stearin	$C_{57}H_{110}O_6$ (890)	0	95.73	76.85	12.36
Found in "mannitol stearin"	—	2.2	82.44	73.1	11.26

What was true of the original "mannitol olive oil" is equally true, *mutatis mutandis*, of "mannitol stearin," which from the above therefore appears to consist mainly of the di-stearates of mannitan and isomannide. We have to record with regret that we have not yet been able to obtain direct evidence of the presence of mannitan and isomannide residues in either product and feel doubtful whether an examination of the products of hydrolysis would lead to very conclusive results.

SUMMARY.

1. By distillation of olein or stearin with mannitol under reduced pressure in presence of a little sodium ethoxide, nearly the whole of the glycerol present in the original fatty compound is expelled.

2. The maximum yield of glycerol is reached when the proportion of fat to mannitol corresponds with two molecules of fat to three of mannitol.

3. The other main products are in both cases water with a little alcohol and a substance which is similar in many properties to the original fat.

4. The composition of the product, whether made with the above proportions of fat and mannitol or with excess of mannitol, corresponds with that of a mixture of the di-oleates (or di-stearates) of mannitan and isomannide. This closely corresponds with the observations mentioned under (2) above.

Professor J. C. Irvine has very kindly confirmed the description we submitted to him of our original mode of preparing glycerol and "mannitol olein" from olive oil. The extension of the work with sugars (and their derivatives other than mannitol) has been left in his hands.

This investigation was carried out for the Food Investigation Board (Dept. of Scientific and Industrial Research) who defrayed the expenses involved.

REFERENCE.

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XXXI. THE DIRECT REPLACEMENT OF GLYCEROL IN FATS BY HIGHER POLYHYDRIC ALCOHOLS. PART II. THE VALUE OF SYNTHETIC MANNITOL OLIVE OIL AS A FOOD.

By

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(Received August 18th, 1919.)

IN the preceding communication Lapworth and Pearson [1919] have described the preparation of a synthetic oil by replacing glycerol in olive oil by mannitol. As part of the general enquiry it was also necessary to ascertain what value, if any, this synthetic product possesses as a food-stuff. A sample of the oil was therefore submitted to us for the purpose of making feeding tests. The product supplied was a light greenish brown oil of somewhat dirty appearance, containing a small amount of solid matter in suspension. The amount of this solid matter increased when the oil was kept standing during the cold weather, and from a cursory examination it appeared to consist of the higher melting point esters (stearin). The oil possessed a taste and odour reminiscent of olive oil, but the former was if anything less pleasant than that of the natural oil.

The following data were obtained on analysis, and for comparative purposes the figures obtained on the analysis of a good quality commercial olive oil are also given. The latter was not, however, identical with the olive oil from which the synthetic oil was prepared.

	"Mannitol olive oil"	Olive oil
Free fatty acids, calculated as oleic acid	4.0 %	2.7 %
Saponification value 	159	193
Iodine value 	82.4	84.7

The investigation of the food value of the mannitol oil was planned to determine first, whether the oil could be assimilated by the mammalian digestive system, and secondly, whether the oil could be administered with safety over considerable periods without any deleterious influence on health. Unfortunately, the amount of material at our disposal was small, and we were obliged to abandon the idea of experiment on man or the higher animals. Such experiments would of necessity have been of very short duration and

quite unsuited to the purpose of the investigation. Accordingly we confined our attention to a study of the absorption of the oil in the digestive tract of the rat, an animal whose suitability for prolonged tests of this type is unquestioned.

As far as we have been able to ascertain, Bloor is the only investigator who has studied the digestibility of mannitol esters of the higher fatty acids in the animal body. He prepared mannide distearate and mannitan distearate and demonstrated that they were hydrolysed by the lipolytic enzymes of the pancreas [1912, 1]. He also carried out feeding experiments of short duration on cats and established the fact that the esters were absorbed from the intestinal tract. One cat received a daily ration of 3.8 g. of mannitan distearate in 5 g. of cotton-seed oil for a period of six days, and showed an absorption of 42.3 per cent. A higher absorption (53.6 per cent.) was observed in an experiment of one day when a much larger amount of the ester was administered. Experiments with isomannide distearate (M.P. 61.5°) showed that approximately 72 per cent. was absorbed, a utilisation comparable with that recorded for tristearin, a natural fat with a melting point of the same order [Arnschink, 1890]. Even better absorbed was isomannide dilaurate [Bloor, 1912, 2], for which coefficients as high as 95—97 per cent. were obtained.

FEEDING TESTS WITH "MANNITOL OLIVE OIL" ON RATS.

To make the tests of the edibility of the mannitol oil as severe as possible it was decided to administer it as the sole source of fat in the diets of the experimental animals. Such a decision introduced a disturbing element into the experiments, for it would necessitate the animals being deprived of the fat-soluble accessory factor, which for several reasons we knew must be absent from the synthetic oil. It was hoped, however, to surmount this difficulty as well as possible by employing animals which were nearly mature. Such animals may live without any apparent disturbance of health upon a diet deficient in fat-soluble A for several weeks [Drummond, 1919]. Two pairs of healthy young adult male rats were selected and confined in suitable metabolism cages. For a preliminary period both pairs were fed upon an artificial diet containing butter fat, after which they received diets containing either olive oil itself or the synthetic mannitol oil for further periods, usually of four days each.

The dietaries were compounded as follows:

	A	B	C
Purified caseinogen	20	20	20 parts
„ starch	50	50	50 „
Yeast extract	5	5	5 „
Salt mixture	5	5	5 „
Orange juice	5	5	5 „
Butter fat	20	—	— „
Olive oil	10	30	— „
Mannitol oil	—	—	30 „

These diets were stiff pastes which were not readily scattered about by the animals during feeding. Every morning fresh water and a ration of diet were placed in the food receptacles in the cages, and the unconsumed diet from the previous twenty-four hours was taken out and weighed. The nitrogen balance was determined in order to follow the general nutritive condition of the animals. Fat was estimated in the food mixtures and faeces by prolonged ether extraction in a Soxhlet apparatus, after dehydration at 110° for twenty-four hours. It was originally proposed to attempt an estimation of the unabsorbed mannitol oil by a polarimetric method, as was done by Bloor [1912, 2], but it was ascertained that the low specific rotation of the oil ($[\alpha]_D^{20} = -1.44^{\circ}$) rendered such a procedure out of the question.

Tables I and II contain the detailed experimental results. The animals all tended to lose weight when receiving the mannitol oil diet, and their food consumption was not good. Rats 3 and 4 both appeared in somewhat poor condition at the termination of their period of 11 days on the mannitol diet. In all cases, however, the absorption of the mannitol oil was good. From the various utilisation values recorded the following approximate averages have been obtained.

Percentage utilisation	
Butter fat and olive oil	97.8
Olive oil	96.6
"Mannitol" olive oil	95.8

From the point of view of the edibility of the mannitol oil it was important to determine whether the slight decline in health which was apparent in the experimental animals on the mannitol oil dietaries was due to some deleterious action of the oil *per se*, or to the fact that they were being maintained on a ration deficient in one of the indispensable accessory factors. To test this point a batch of young rats of uniform age and sex were selected and divided into two sets. One of these received a diet containing mannitol oil together with butter fat as a source of fat-soluble A (Diet E), the other received a diet containing equivalent amounts of natural olive oil and butter fat (Diet F). The composition of the two rations was as given below:

	Diet E	Diet F
Purified caseinogen	20 parts	20 parts
.. starch	50 "	50 "
Yeast extract	5 "	5 "
Orange juice	5 "	5 "
Salt mixture	5 "	5 "
Butter fat	10 "	10 "
Olive oil	— "	10 "
Mannitol oil	10 "	— "

Unfortunately the experiments were restricted by the small amount of mannitol oil which was available. The results which were obtained are tabulated in Table III.

Table III. *Average body weight in grams of two groups of six male rats.*

Days	Diet E Mannitol oil- butter diet	Diet F Olive oil- butter diet
0	64	67
7	72	85
14	94	98
21	106	112
28	121	132
35	136	148
42	153	162

On the whole, the growth of the rats which received the butter-fat-olive-oil mixture was more satisfactory than that of the animals on the mannitol oil diet. The difference in the final weights is not very marked, and it is difficult to express an opinion as to whether it is of any significance, particularly in view of the small number of animals employed.

At the conclusion of the experiment the mannitol-fed animals appeared in quite as good condition as the control lot, so that it appears safe to assume that no toxic factor was present in the sample of synthetic mannitol olive oil. That this oil was less palatable than olive oil appears likely, however, from the fact that the food consumption of the batch on Diet E was appreciably less than of that receiving Diet F.

The importance that was attached to this investigation in the period during the recent war, when urgent demands of fat for glycerol were bringing about a drastic reduction in the amount available for edible purposes, is fortunately now no longer so great. The results are therefore placed on record purely in the light of their scientific interest.

SUMMARY.

1. A synthetic oil prepared by replacing glycerol in olive oil by mannitol is utilised by the animal organism to practically the same extent as olive oil itself.

2. No toxic action was found to follow the prolonged administration of this oil to rats.

This investigation was carried out for the Food Investigation Board (Dept. of Scientific and Industrial Research), and the expenses involved were defrayed out of a grant from the Royal Society.

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XXXII. RELATIVE ANTI-SCORBUTIC VALUE OF FRESH, DRIED AND HEATED COW'S MILK.

BY ROSAMUND EVELYN BARNES AND ELEANOR MARGARET HUME.

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(Received August 19th, 1919.)

- (1) Experiments with guinea-pigs by R. E. Barnes.
- (2) Experiments with monkeys by E. M. Hume.

INTRODUCTION.

IN two previous communications [Chick, Hume and Skelton 1918, 1, 2] one of us (E. M. H.), in conjunction with other workers in this Department, has shown that raw cow's milk must be classed among the less valuable foodstuffs as regards anti-scorbutic properties. It was shown, for example, that whereas in case of many raw fruits and vegetables amounts varying from 1.5 to 10 g. daily will protect guinea-pigs from scurvy upon a diet otherwise devoid of all anti-scorbutic material, 100—150 cc. of raw cow's milk was needed to give similar protection. The opinion was also put forward that dried milk was even less valuable than raw milk in respect of anti-scorbutic properties; this decision was reached mainly on theoretical grounds—there being at that time few experimental results available. But it had been shown that sundry other foodstuffs, rich in the anti-scorbutic vitamine, lose the greater part of this principle when no longer associated with a living tissue, as, for example, when the foodstuff is dried or preserved by other means. As regards dried milk, this view is in opposition to that expressed by many medical authorities, who, on the basis of their clinical experience, consider that dried milk is in every respect a satisfactory substitute for fresh milk and may be adopted as a sole diet for children without any risk from scurvy [see Coutts 1918, Naish 1914].

The present paper deals with an attempt to gain by direct experiment a quantitative estimate of the anti-scorbutic value of dried milk compared with raw milk. The first series of experiments was made with guinea-pigs by one of us (R. E. B.) and the details of the work are set out in the next chapter.

The methods and technique were the same as those employed in the previous work already mentioned. Guinea-pigs are however not well suited for work upon the anti-scorbutic value of milk. In order to maintain health

and to prevent scurvy, these animals need a comparatively large amount of anti-scorbutic material in their diet; in case, therefore, of a foodstuff like milk with a low content of anti-scurvy vitamine, it is necessary for large quantities to be consumed. It is against the habit and nature of these animals to take much liquid and we have never come across an animal which would take voluntarily the large daily ration (100 cc. and upwards) of raw milk necessary to afford protection from scurvy. Hand feeding of these large quantities is indescribably tedious and in many cases they cannot be tolerated without digestive disturbance. The results obtained in a few successful cases out of a large number of trials are given below (Table I), together with those obtained by similar methods with dried milk. The inferiority of the latter as regards anti-scorbutic properties is clearly shown.

The importance of this result in relation to the artificial feeding of infants made it very desirable to repeat the experiments with a second experimental animal. Young, growing monkeys were selected, and the work was carried out by E. M. H. Many monkeys are extremely fond of milk in any form, and once the right species and individual have been selected, there is little difficulty in persuading the animal to take the daily ration ordained. Symptoms of scurvy in the monkey are more closely allied to those of the human disease than is the case with guinea-pig scurvy, and in these young monkeys appeared to be identical with those of infantile scurvy. The results obtained were in complete accord with those obtained from the work with guinea-pigs. The quantity of milk which, added to a diet containing no other anti-scorbutic material, successfully prevented the onset of scurvy when given in the raw condition, failed to avert it when prepared from dried milk; and, further, the scurvy symptoms developed in the latter case could be cured if the dried milk ration was substituted by an equal amount of raw milk.

In these experiments with monkeys, evidence was obtained that the anti-scorbutic value of cow's milk (and of the dried milk prepared from it) was dependent to some degree upon the season of the year, summer milk, from May onwards, being richer in anti-scorbutic properties than winter milk (see below, p. 316). This fact is doubtless to be attributed to the difference in the cow's diet at these different seasons, the winter diet of mangolds, hay, cotton cake and cereals being poor in anti-scorbutic vitamine when compared with the summer diet of fresh greenstuff.

The bearing of these results upon the problems connected with infant feeding will be discussed later on, when the experimental work has been described in detail.

EXPERIMENTS WITH GUINEA-PIGS, BY R. E. BARNES.

Raw Milk.

The fresh milk used for these experiments and for those with monkeys, described below, was obtained from a model dairy and was very pure, specially delivered country milk.

The successful trials with raw milk are comparatively few for the reasons given above. The results of six experiments set out in Table I *a* afford, however, useful confirmation of similar work described in the previous communication referred to above [Chick, Hume and Skelton 1918, 1]. The diet, as then, consisted of oats, wheaten bran and fresh cow's milk, and it was again found that scurvy could be prevented and health maintained in those cases in which 100—150 cc. were taken.

The symptoms of guinea-pig scurvy and the post-mortem appearances have been described in previous communications [Chick, Hume and Skelton 1918, 2; Delf and Tozer 1918] and it is unnecessary to recapitulate them here.

Of these six experiments made with raw milk, the cream was removed in three cases by simple centrifuging. Milk thus separated seemed to agree with the animals better than full cream milk, and fewer animals were rejected after trial. As regards the anti-scorbutic value, no evidence was obtained that this treatment had any marked influence.

Dried Milk.

The dried milk used was a well-known commercial brand prepared by the Hatmaker process. In this process milk is poured in a thin film over steam-heated rotating cylinders, the water of the milk is rapidly evaporated and the solids left in a thin skin on the surface of the drums. After the drum has made about two-thirds of a revolution this skin is scraped off with knife blades, broken up and passed through a sieve as a finely granular powder. The temperature of the revolving drums is considerably over 100°, but the time of contact is stated by the manufacturers to be a few seconds only.

Two varieties of this dried milk were obtained for these experiments with the kind cooperation of the manufacturers. In one set of experiments the dried milk was quite fresh, being delivered at the Institute less than one week after manufacture, and used for experiment about one week later (Table I *b*). The second variety was imported from a distant colony and was at least six months old when received, and probably not less than nine months. It must therefore have been nearly one year old before the conclusion of some of the experiments (Table I *c*).

The results obtained gave no indication that the age of the product had much effect upon its anti-scorbutic value, but the experiments were not calculated to show any fine shades of difference. Against the greater age of this second sample of Colonial origin, must however be set the fact that the

Table I. (a) *Anti-scorbutic value of raw cow's milk; experiments with guinea-pigs.*
The basal diet consisted of 10-20 g. oats and wheaten bran mixed.

No. of animal	Average amounts of milk consumed daily, in successive periods of 23 days,				Date experiment began	Length of experiment, days	Weight of animals in g.			Result
	1st	2nd	3rd	4th			Initial	Highest reached	Final	
*675	91.8	76.7	—	—	Aug. 12th, 1918	46	360	446	330	- 8.3 Death from unknown cause; traces only of scurvy found post-mortem; no symptoms of scurvy during life
*676	90.1	109.9	128.2	—	Aug. 12th, 1918	91	356	583	580	+ 60.2 In good health all through experiment—no scurvy
*680	103.6	103.3	112.2	—	Oct. 28th, 1918	87	353	577	458	+ 29.7 Death from disease—no evidence of scurvy post-mortem
†670	129.8	88.3	—	—	July 10th, 1918	36	365	466	280	- 23.2 Death from scurvy
†674	79.4	133	161.5	—	July 24th, 1918	89	334	543	442	+ 32.3 Animal in good condition. Slight signs of scurvy post-mortem
†681	98.1	119.3	134.9	147.2	Aug. 12th, 1918	111	340	474	474	+ 39.4 Animal showed signs of scurvy from 22nd day, improved from 44th day, recovered and enjoyed good health until end of experiment. Post-mortem—signs of old scurvy

(b) *Anti-scorbutic value of dried cow's milk, 1-2 weeks after manufacture.*

660	118.1	97.3	—	—	June 3rd, 1918	43	282	373	222	- 21.2 Scurvy the main cause of death; slight infection as well
661	82.1	86.8	—	—	May 31st, 1918	48	278	293	176	- 36.7 Death from scurvy, lesions very severe
668	98.7	78	—	—	July 2nd, 1918	32	342	406	298	- 12.8 Death from severe scurvy
677	92.5	—	—	—	Aug. 18th, 1918	25	360	410	286	- 20.5 Animal wretched, chloroformed—marked scurvy
683	106.4	70	—	—	Nov. 4th, 1918	30	348	396	266	- 23 Death from scurvy, though lesions not very severe

(c) *Anti-scorbutic value of dried cow's milk, 6-9 months after manufacture.*

648	88.5	102.4	112.5	—	—	67	341	414	282	- 17.3 Scurvy; signs in ribs only
649	97.5	120	93.1	—	—	71	306	400	238	- 22.2 Death from scurvy
650	90.1	104.4	112.1	—	—	70	322	399	300	- 6.8 Death from chronic scurvy
651	92.7	100.1	115.1	—	—	72	318	380	239	- 24.8 Wretched condition; chloroformed—distinct scurvy
653 A	82.	88	—	—	—	33	301	360	220	- 26.9 Very ill, chloroformed—severe scurvy

* Full cream.

† Separated.

Colonial cows, so the manufacturers stated, are grass fed all the year round. It is probable therefore that this Colonial dried milk had a much greater anti-scorbutic value to start with and even after six months may still have contained more than milk from stall fed cows even when freshly manufactured. Out of a total of five animals on each type of dried milk, not one escaped scurvy, although one animal in each series showed slight signs only (Nos. 648 and 683). On the whole, those receiving the older material lived longer, suggesting a greater reserve of anti-scorbutic vitamine, but the scurvy lesions, post-mortem, were correspondingly more severe.

When these results are compared with those obtained with raw milk, a great difference in the anti-scorbutic value is at once evident. In those cases where a large enough ration of raw milk was consumed, scurvy was prevented and in cases like 681 (Table I *a*) where the average amount consumed for the first 28 days of the experiment, 98 cc., was not enough to protect the animal, the larger rations consumed afterwards (119 cc. daily for a second period of 28 days and 135 and 147 cc. respectively for two succeeding similar periods) caused an improvement in the symptoms, and effected a successful cure. In case of the animals on dried milk no such effect was noticed. The amounts consumed (reckoned in terms of raw milk) were in many cases not less than those which successfully prevented scurvy in case of raw milk, and some experiments show a close parallel in the quantity actually taken. For example, No. 676 (Table I *a*) on raw milk enjoyed good health throughout while No. 650 (Table I *c*) on dried milk died from scurvy and these two animals consumed very similar amounts during the three periods of 28 days, into which the experiments were divided. In case of No. 676, these amounts were 90 cc., 110 cc. and 128 cc. and in the case of No. 650, 90 cc., 104 cc. and 112 cc. respectively for the three periods. It was usual for the appetite to fall off with onset of scurvy symptoms, and the usual increase in the amount of milk taken with increasing age of the animal, which is very noticeable in many of the raw milk experiments, is not apparent in case of dried milk.

GROWTH PROMOTING PROPERTIES OF RAW AND DRIED MILK.

The four curves in Fig. 1 show growth of four animals receiving respectively full cream raw milk (A); separated raw milk (B); freshly manufactured dried milk (C); dried milk 6—12 months old (D); normal diet of cabbage and grain for comparison (N). Those animals were selected for comparison whose consumption of milk was as nearly as possible the same. During the first 20—30 days of the experiment, the degree of growth is similar in all cases; with the onset of scurvy symptoms, there is decline and failure to grow in case of the animals receiving dried milk.

This failure is satisfactorily explained by the onset of scurvy and it is not necessary to assume that the growth promoting vitamins, water-soluble B, or fat-soluble A, have suffered serious damage in the process of drying. If

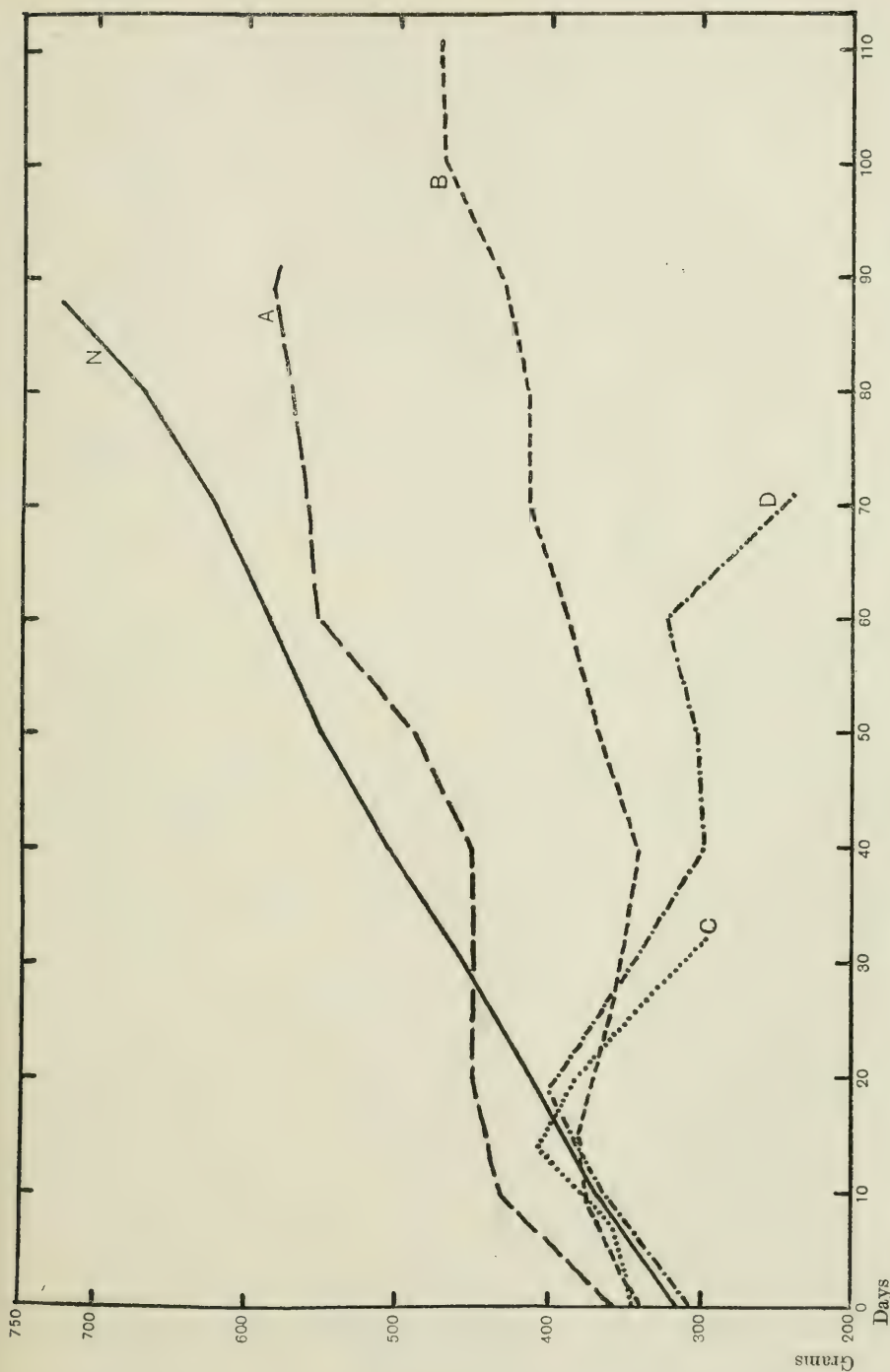


Fig. 1. Weight curves of guinea-pigs upon diet of oats, bran and milk of various types, the quantities taken being roughly equivalent.
 ——— N. Average weight curve on diet of oats, bran and cabbage leaves.
 - - - - - A. Full cream raw milk. No. 676. No scurvy.
 B. Scurvy developed. No. 668. Scurvy developed.
 - · - · - C. Dried milk (English) 1—2 weeks after manufacture. No. 681. Slight scurvy cured later.
 - - - - - D. Dried milk (Colonial) 6—9 months after manufacture. No. 651. Scurvy developed.

there had been any defect in the diet from this cause, the failure to maintain growth in case of dried milk would have been evident from the beginning of the experiment, whereas it was only evident after sufficient time had elapsed for the lack of anti-scorbutic material to make itself felt. It must be admitted that in case of the experiments with raw milk, owing to the size of the ration, the fat-soluble factor was probably present in large excess of the guinea-pig's requirements, and that any loss occurring during the drying might not have been sufficient to bring it below this level.

This point seemed of sufficient importance to warrant further investigation, and special experiments are now in progress to determine the relative value, as regards growth, of raw milk, dried milk and strongly heated milk respectively. In this work, the results of which will be published shortly, the quantities fed to the animals were considerably reduced, in order to determine the amounts which, in the several cases, will limit growth in guinea-pigs upon diets arranged to be satisfactory in all other respects. The results at present obtained show no significant difference between the growth promoting properties of equivalent amounts of dried and fresh milk.

Note.

While the present paper was in course of preparation two articles appeared by Hess and Unger [1919] and by Hart, Steenbock and Smith [1919] upon which comment is necessary.

Hess and Unger [1919, pp. 295, 296] find that 80 cc. daily of fresh milk or the equivalent dried by the Just-Hatmaker process are sufficient to cure guinea-pig scurvy which has already developed. This result is apparently in conflict with our experiments in which 100—150 cc. raw milk were found necessary to prevent guinea-pig scurvy and protection with dried milk was not attained at all. The reason for this discrepancy is doubtless to be found in differences in the basal diets adopted by these workers and ourselves. We employed oats and bran only, while Hess and Unger included hay *ad libitum*. Delf and Skelton [1918] have shown that vegetable leaves (cabbage) lose a great proportion, but not all, of their anti-scorbutic value when dried, and that the loss varies according to the length of time which has elapsed since the drying took place. Hay must therefore be looked upon as not entirely devoid of anti-scorbutic properties which will vary according to its age, probably also according to its composition. If fed *ad libitum*, a third variable is introduced, in the difference between the appetite of individual guinea-pigs and consequently in the amount consumed.

In experiments destined to estimate the anti-scurvy value of any particular food stuff, *e.g.* milk in this instance, the foodstuff in question should constitute the only variable in the diet; if hay *ad libitum* is given with the basal diet, two and possibly three, variables are included in addition.

The inclusion of anti-scorbutic material in the basal diet makes it possible to protect guinea-pigs from scurvy with smaller rations of milk than could

otherwise be effected, and by such a procedure it might be possible to obtain a quantitative estimate of the comparative value of dried compared with raw milk, which could not be obtained by our method.

But the results of such experiments can only be regarded as trustworthy when the included material is standardised both as regards its own anti-scorbutic value and the quantity consumed by the guinea-pigs.

It is stated by Hess and Unger [1919, p. 295] that Chick and Hume and other workers at the Lister Institute have used dried milk as part of their basal dietary—"because it was found that this milk was devoid of anti-scorbutic power." We have never used dried milk as part of our basal diet in this Institute, nor do we regard it as devoid of anti-scorbutic power as the present paper shows clearly. The milk which has been used regularly in our basal diet has been autoclaved at 120° for one hour and given in constant quantity, 60 cc. daily to each guinea-pig. In milk so treated we have been unable to find any significant survival of anti-scorbutic value.

In the paper by Hart, Steenbock and Smith [1919] to which attention is also drawn, hay *ad libitum* was again included in the basal diet of the guinea-pigs and the same criticism applies. They too obtain protection from scurvy with rations of milk considerably smaller than those which we have found necessary: in the case of fresh milk a daily ration of 84 cc. gave complete protection from scurvy and partial protection was obtained with 30 cc. daily. The authors themselves [1919, p. 308] suggest the probability that "the hay still contains some of the anti-scorbutic vitamine, which in addition to that in the milk and grain is sufficient to prevent the development of the disease" on a ration of fresh milk smaller than would be needed to protect when no hay is given.

EXPERIMENTS WITH MONKEYS, BY E. MARGARET HUME.

Scurvy in monkeys has been described by Hart and Lessing [1913], Harden and Zilva [1918], and Chick, Hume and Skelton [1918, 3]. When these animals are fed upon a diet deficient in anti-scorbutic material but otherwise adequate, scurvy is developed in about ten weeks [Harden and Zilva 1918], with symptoms closely resembling those of human scurvy. Such are sponginess and bleeding of the gums with loosening of the teeth, and haemorrhages under the periosteum and in almost any part of the body. There is weakness and ultimately almost complete powerlessness of the hind limbs, a condition characteristic of infantile scurvy which has been called "pseudo-paralysis" [Barlow 1894], and which is apparently the result of haemorrhages into the hip and knee joints. The bones also become fragile and there is enlargement and disruption of the rib-junctions. When the disease is far advanced the whole body and limbs are tender.

Barlow ascribes the sub-periosteal haemorrhage in scurvy of infants to an increased vascularity of the periosteum from which blood is assumed to

escape and to collect between the periosteum and the bone. From post-mortem examinations of slight and incipient scurvy in monkeys it has seemed rather as if the bone became rarified at an early stage of the disease and that blood oozed outwards through the bone, presumably from haemorrhage in the bone marrow.

Alterations in the gums were observed to occur earlier and to be much more severe in monkeys which were changing their temporary for their permanent dentition; the onset of scurvy arrested the eruption of the teeth, the gums became swollen and blood blisters were frequently formed over the site of the budding tooth. Gum symptoms were usually the first sign of scurvy to be observed, but occasionally the weakening of the hind legs preceded them. This so-called "pseudo-paralysis" is very characteristic. At first only a slight decrease in activity is noticed, the animal does not jump as high or as carelessly as it was wont to do; this condition progresses until at last the hind legs become useless and the animal swings itself about in a sitting posture by the aid of its fore-limbs.

The effect of scorbutic symptoms upon the weight of the monkey is similar to that observed in case of guinea-pigs. About the time of the first appearance of scurvy symptoms, the animal ceases to gain in weight and soon afterwards a slow but gradually accelerated loss of weight begins (Fig. 3, curve A, p. 321). If a cure is then administered, this loss is soon checked and after a short period of maintenance weight is again put on at a remarkable rate (Fig. 3, curve A, p. 321). The original weight of the animal is regained and the normal rate of growth, if the animal is young and growing, is re-established.

The monkeys used in this set of experiments were not very numerous and were of various kinds and sizes. All were young, growing, healthy animals at about the age of the second dentition, probably, therefore, from one-and-a-half to three years old. They included the genera *Macacus* (two species), *Cercopithecus* (two species) and *Cercocebus* (one species). As the animals were so few and so various, special precautions had to be taken to avoid error due to idiosyncrasy. The method pursued was, in the first place, to determine the minimum dose of fresh and dried milk respectively, which would protect a monkey from scurvy when on a diet otherwise devoid of anti-scorbutic material. In the second place, when scurvy had developed upon a certain daily ration of dried milk, cures were attempted by substituting the same sized ration of raw milk. This procedure eliminated many sources of error, in that the comparison between the two foodstuffs was carried out upon the same individual animal.

The basal diet. The staple item was boiled polished rice, of which 100 g. (dry weight) was allowed daily to each animal; to this was added 30 g. daily of raw wheat germ to provide an abundant supply of the water-soluble B or anti-beriberi factor. A piece of wheaten biscuit, a few pea-nuts or dried peas or a little paddy rice were given as a relish. To the basal diet was added

the measured ration of milk, warmed very slightly and heavily sweetened; this served as the source of the anti-rachitic and fat-soluble A factor and was also the only source provided of the anti-scorbutic factor. When the ration was below 200 cc. a day it was made up to this amount by addition of milk autoclaved at 120° for one hour to deprive it of anti-scorbutic vitamin. This addition was made in order to increase the amount of anti-rachitic factor in the diet; the monkeys, however, would not always accept it.

The milk ration was usually divided into two parts and offered to the animals morning and evening, the monkeys being trained to drink out of a dish held in their cages. It was occasionally necessary to feed with a pipette, syringe or spoon if the animal grew tired of the milk and had to be coaxed to carry the experiment through.

The milk used for the experiments with raw milk was the same as that used in the work with guinea-pigs (p. 308). The dried milk was also of the same brand as in the foregoing experiments, but the work was confined to the English material of recent manufacture; in some trials it was one to two weeks old and in others two to three weeks old.

A summary of the results obtained is set out in Table II and full protocols of all the experiments are given later, p. 320. They include three trials with dried milk and six trials with raw milk. One of the latter, Exp. 1 *A*, was a curative experiment following a period on dried milk during which definite and severe scurvy was developed.

As regards the amount of raw milk which must be consumed daily to prevent scurvy in monkeys, rations of 50 cc. and 75 cc. proved too small in case of two monkeys, David (initial weight 2080 g.) and Diana (initial weight 2770 g.) respectively. 125 cc. was found adequate (see Exp. 6, Imp, weight 1940 g.), so also was 200 cc. (Exp. 8, Rags, weight 2230 g.), while 175 cc. cured Jane (Exp. 1 *A*, initial weight 2180 g.) of scurvy which she had acquired on a diet containing an equivalent amount of dried milk.

On the basis of these results we might place the minimum protective ration of raw cow's milk at about 100 cc. daily. The case of the monkey Chirgwin (Exp. 7) who developed slight symptoms of scurvy, but was otherwise in good health for six months upon a daily ration of 150 cc., is somewhat out of line with the above. This particular experiment was of less value than some of the others from the fact that the raw milk experiment followed a period of 43 days on dried milk, after which the nature of her ration had to be changed owing to her sudden, but persistent, refusal to take dried milk.

In the case of dried milk we have definite proof of the inadequacy of amounts equivalent to 175 cc. and 200 cc. milk daily in case of monkeys Jane (Exp. 1) and Toby (Exp. 2) respectively. Both these monkeys developed definite and severe scurvy in from three to four months; and in case of Toby, the ration was increased to 300 cc. without in any way relieving the scurvy symptoms which, on the contrary, became progressively more severe. The third monkey, Caesar (initial weight 2740 g.), received the

equivalent of 250 cc. daily. After about four months his condition suggested most distinctly the onset of scurvy, his ceaseless activity became less marked, his hind limbs became slightly weak and his weight stationary. As the time progressed, his condition improved, however, and it appeared as if his milk ration was increasing in anti-scorbutic value. The change for the better took place about the middle of June, about four weeks after incipient scurvy had been detected, and at a period of the year shortly after the time when cows change their winter stall feeding for the open pasture. Such an alteration in diet may be expected to influence the anti-scorbutic value of the milk given by the cow and also of the dried milk prepared therefrom, especially when, as was the case in these experiments, it is consumed very shortly after manufacture. A disturbing factor is thus introduced into the experimental work, when the series of experiments extends over one year as was the case in the present research. The milk was consequently derived from cows which during a portion of the time were stall-fed and during other portions were pasture fed. The milk, either dried or fresh, cannot therefore be regarded as a uniform article throughout the period. It is judged, however, that this fact did not materially alter the trend of the experiments, seeing that trials with raw and dried milk went forward simultaneously, and the latter was given so soon after manufacture. In the case of the monkeys Caesar and Chirgwin (see below, p. 323), however, the taking of the cows out to pasture about the middle of May was doubtless the means of raising a ration of dried milk of 250 cc. (Caesar) and a ration of fresh milk of 150 cc. (Chirgwin) from a non-protective to a protective amount.

The influence of differences in the cow's diet upon the anti-scorbutic value of the milk given makes it, however, impossible to obtain an absolute standard for the anti-scorbutic value of cow's milk and it is only possible to say that *the daily ration of raw milk which protects from scurvy a monkey of 2-3 kg. weight is from 125 to 175 cc., while the corresponding ration of dried milk, of fresh manufacture, is from 250 to 300 cc.*

It is interesting to note that the amount of raw milk needed to protect a guinea-pig from scurvy, 100 to 150 cc., is almost as great as that needed to protect a monkey. The corresponding amount of dried milk, freshly manufactured, necessary for a guinea-pig, would therefore be the equivalent of well over 200 cc., an amount far too large to be consumed by an animal of 300 to 400 g.s weight. It is not, therefore, surprising that all efforts to protect guinea-pigs from scurvy with dried cow's milk, even when given in a concentration greater than the normal, should have proved unsuccessful.

EXPERIMENT WITH HEATED MILK.

Only one experiment was made in which the anti-scorbutic value of raw cow's milk, scalded, was tested.

The experiment was designed to show whether milk, heated to a degree

which is commonly believed to destroy the bacillus of tuberculosis, suffers serious loss in anti-scorbutic vitamine.

The milk was heated quickly in an enamel pan over a Bunsen burner; it was removed at the moment when it frothed up, at the boil, and was allowed to cool in the air. The total period of heating was found to be about four minutes, during which time the milk was between 70° and 100° for about one-and-a-half minutes. After the pan was lifted from the flame, four minutes elapsed before the temperature of the milk fell again to 70°, and a further 17 minutes before it reached 40°. The whole operation consisted therefore in exposure to a temperature between 70° and 100° for about five-and-a-half minutes.

A ration of 200 cc. of such milk was given to a monkey (Toby) which had developed scurvy on a daily ration of 200 cc. of dried milk. The cure was rapid and complete.

The loss in anti-scorbutic vitamine is therefore not as great as is the loss suffered by milk in drying, but since the minimum amount of raw milk needed to protect a monkey is less than 200 cc., being about 150 cc., the milk might, in the scalding, have suffered loss to the extent of 25 per cent. of the anti-scorbutic vitamine it originally contained, without its being possible to detect such loss by the one experiment made. A loss of more than 25 per cent. would however have reduced the anti-scorbutic value of the ration to less than that of 150 cc. raw milk, but the rapidity of the cure suggested that the animal was receiving a considerable excess over and above the minimal ration needed to protect. Such scalding may therefore be looked upon as a means of sterilisation causing comparatively little damage to the anti-scorbutic vitamine.

GROWTH PROMOTING POWERS OF RAW, DRIED AND HEATED MILK.

None of the experiments described was specially designed to test the growth promoting powers of the milks used, but as in most cases the milk ration was, at the same time, the chief, if not the sole source of the fat-soluble A (anti-rachitic) growth promoting factor, it is possible to draw certain deductions. These are necessarily somewhat rough owing to the diversity in kinds and ages of the monkeys used.

It was found that a ration of 200 cc. daily of milk of any description, raw, dried, scalded or autoclaved, gave good growth but where smaller quantities were used growth was retarded.

In the post-mortem examination of the monkey, David (Exp. 4), which received 50 cc. or less of raw milk, distinct symptoms of rickets were observed. The rib junctions were enlarged and there were also secondary enlargements on the bony shaft of the rib, enclosing cartilaginous islands (see Fig. 2), such as are so frequently seen on the ribs of monkeys kept long in confinement on unsuitable diet in zoological gardens. This monkey although

Table II. *Anti-scorbutic value of raw, dried and heated cow's milk. Experiments with monkeys.*

No. of exp.	Period of year	Name of animal	Average daily ration, cc.	Time of exp. days	Time after which onset of scurvy noted, days	Initial weight of animal g.	Change in weight during course of experiment, %	Remarks
1	July 1918 to December 1918	Jane (<i>Macacus cynomolgus</i>)	175	142	105	2090	+ 4	Cured with equivalent amount of raw milk before any great loss in weight had occurred (Exp. 1 A)
2	Dec. 1918 to March 1919	Toby (<i>Cercopithecus fuliginosus</i>)	1st period 200 2nd period 300	97 7	87 continuous	2470	+10.5	Cure attempted with 300 cc. dried milk, symptoms worse after 7 days (see also Exp. 2 A)
3	Feb. 1919 to July 1919	Caesar (<i>Cercopithecus callitrichus</i>)	250	161	117 but disappeared later	2740	+ 15.5	Scurvy symptoms observed on 117th day, remitted later about time when cows go to pasture
1 A	Dec. 1918 (1) to Feb. 1919, (2) to July 1919	Jane (<i>Macacus cynomolgus</i>)	1st period 175 +2nd period 125	54 162	— —	2180 2450	+12 14	Successful cure of scurvy developed on similar ration of dried milk Excellent health and growth
4	July 1918 to Dec. 1918	David (<i>Macacus rhesus</i>)	50 (maximum value)	150	66	2080	-40	Scurvy developed on full ration of 50 cc. daily, later part of ration refused; died from dysentery, signs of mild scurvy and rickets at post-mortem examination
5	July 1919 to Dec. 1918	Diana (<i>Macacus rhesus</i>)	75	143	119	2770	+ 7	Gained in weight until onset of scurvy, refused part of ration with increase in severity of symptoms; successful cure with 10 g. daily germinated peas
6	Dec. 1918 to July 1919	Imp. (<i>Cercopithecus sabaeus</i>)	+125	182	—	1940	+ 20	Excellent health and fair growth
7	Jan. 1919 to July 1919	Chirwin (<i>Cercopithecus sabaeus</i>)	150	184	115 (diminished later)	2770	+10.5	Previous to experiment received ration of dried milk equivalent to 200 cc., imperfectly taken, for 43 days. Scurvy symptoms on 115th day but much mitigated when cows go to pasture
8	Dec. 1918 to July 1919	Rags (<i>Cercopithecus fuliginosus</i>)	200	225	—	2230	+ 50	Excellent health and growth
2 A	March 1919 to May 1919	Toby (<i>Cercopithecus fuliginosus</i>)	200	49	—	2730	+ 11	Successful cure of scurvy developed on dried milk, marked improvement after 7 days (see Exp. 2 above)

* In case of dried milk expressed as the equivalent volume of fresh milk.

+ 75 cc. autoclaved milk added from the 60th day in Exp. 1 A 2nd period, and from 105th day in Exp. 6.

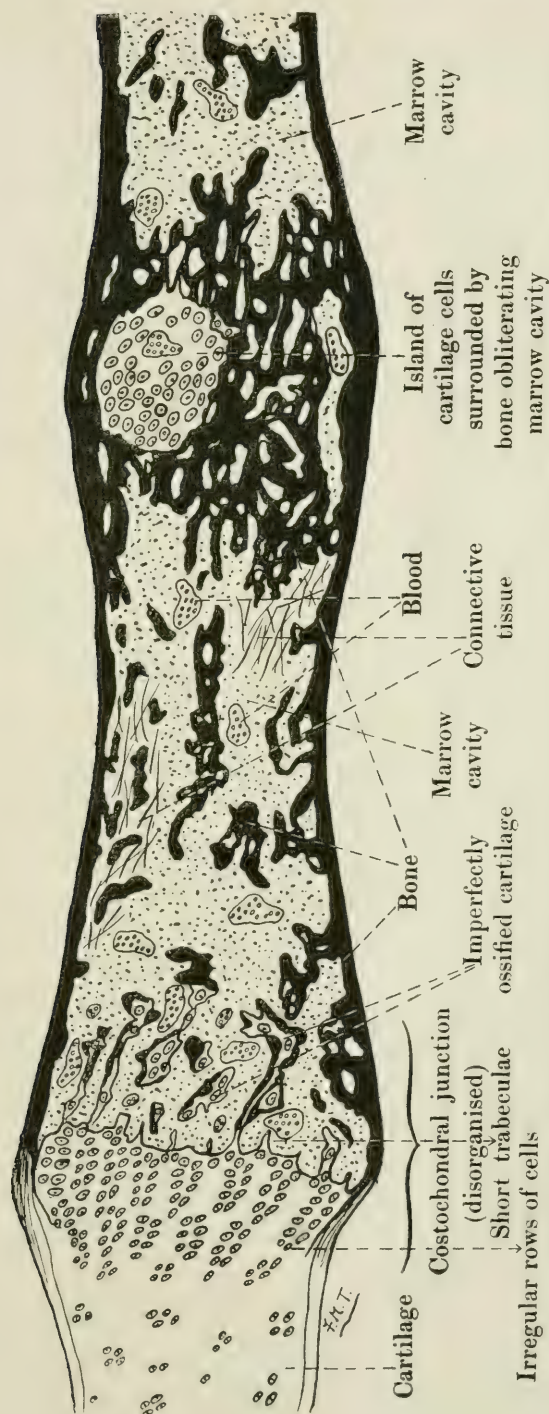


Fig. 2. Diagrammatic representation of a section of a rib junction and secondary nodule believed to be of rachitic origin (Monkey 4 David).

a young and growing one (initial weight 2080 g.) showed a daily increment of 1.5 g. only, up to the time when he developed scurvy. The histology was kindly examined by Miss F. M. Tozer, to whom I am indebted for the drawing reproduced in Fig. 2.

If a comparison is made between the weight curves (Fig. 3) of the monkeys Toby (curve A, 200 cc. dried milk) and Rags (curve B, 200 cc. raw milk), for the first 70 days of the experiment, *i.e.* up to the time when Toby developed scurvy, it is seen that Toby on dried milk shows a daily increment of 6.4 g. while Rags on raw milk shows one of 6.1 g. The rate of growth is therefore almost the same, being slightly in favour of the monkey on dried milk. These two animals are particularly well chosen for comparison as being more nearly alike than any of the other experimental monkeys. Both were male *Cercocebus fuliginosus*, purchased together, presumably of the same past history, and their initial weights, 2470 g. and 2230 g. were also nearly the same.

No two of the other animals are strictly comparable either in themselves or in the ration they received.

Jane (initial weight 2090 g.) over the various periods of her experiment gives the following daily increments:

Daily ration of milk	Days of exp.	Rate of growth, g. per day
Dried milk, 175 cc.	63	4.1
Raw milk, 175 cc. (period of cure) ...	24	2.5
" 125 cc.	60	2.0
" 125 cc.; autoclaved milk, 75 cc.	102	2.2

The only marked superiority here, seems to be in the case of the large ration of dried milk; the large ration of raw milk was not continued sufficiently long to give a conclusive result. In considering these figures it must, however, be remembered that monkeys' growth is not necessarily uniform and conclusions drawn from comparative growth, over consecutive periods, on the same animal may not give reliable results. As far as the experiments go, however, the results obtained with monkeys confirm those obtained with guinea-pigs and show that dried milk is not appreciably inferior to raw milk as regards the growth promoting properties, and the content of the fat-soluble A factor. Experiments with rats [see Coutts 1918; Lane Claypon 1916] are in harmony with this view.

PROTOCOLS.

David. (No. 4, *Macacus rhesus*. Male. Initial weight 2080 g.) Raw milk ration 50 cc. Scurvy developed.

For the first 42 days he refused to drink the milk, it was therefore poured over the cooked rice meal; the amount consumed was consequently less than 50 cc. per diem.

For the next 41 days the milk was taken regularly from the syringe. About the 66th day haemorrhagic spots were noticed on the gums and incipient scurvy was diagnosed. On the 85th day the animal met with an accident, biting its tongue so severely that it refused its milk and never again consumed the full 50 cc. The scurvy symptoms progressed, stiffness of the hind legs

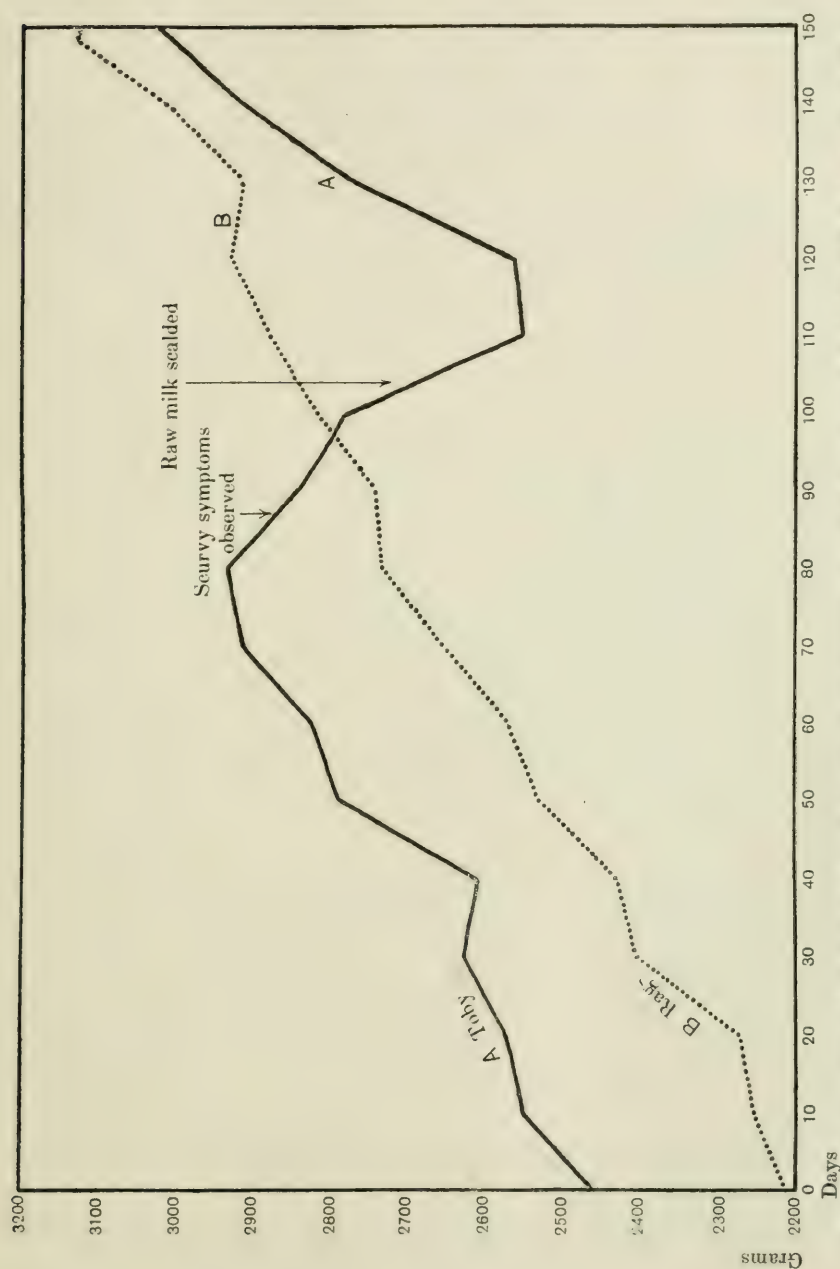


Fig. 3. Weight curves of two monkeys, showing comparison between fresh milk scalded, dried milk, and raw milk. Toby (curve A) received 200 cc. dried milk daily for 104 days, scurvy developed on 87th day. Fresh milk scalded (200 cc.) daily administered from 104th day with complete cure.

Rags (curve B) received 200 cc. raw milk daily throughout and remained in health.

was observed, the animal lost weight and finally severe dysentery set in; he was chloroformed on the 148th day.

The post-mortem examination showed incipient scurvy, haemorrhage into the synovial cavities of knees, elbows and hips, and petechial haemorrhage in the dura mater; there was sponginess at the ends of the long bones and incipient sub-periosteal haemorrhage in the same regions. There was enlargement of the costochondral junctions, probably of rachitic origin, and there were also secondary enlargements along some of the rib bones, nearer to the spine (see Fig. 2, p. 319). The bones of the ribs and skull were much rarified and could be cut through easily with scissors.

The large intestine showed the inner surface mucoid and slightly haemorrhaged, probably from dysentery, but no organism was recovered which corresponded to a typical dysentery.

Scurvy symptoms first appeared when the full ration of 50 cc. of raw milk was being consumed. A moribund state was induced by dysentery supervening, presumably on a condition where the immunity was lowered by incipient rickets and scurvy.

Diana. (No. 5, *Macacus rhesus*. Female. Initial weight 2770 g.) Raw milk, average consumption 75 cc. Scurvy developed.

This monkey consumed on an average 75 cc. raw milk daily from the syringe and on this diet she gained weight slowly till scurvy symptoms supervened. Slight sponginess and haemorrhage of the gums were observed on the 119th day. From this time onward the milk was consumed less well and the scurvy symptoms progressed more rapidly; the gums became markedly spongy and bleeding; the hind legs became uncertain and a haemorrhage about the eye produced the discoloration characteristic of a black eye.

On the 143rd day, anti-scorbutic material in the form of 10 g. daily of raw germinated peas (soaked 24 hours, germinated 48 hours) was administered together with a liberal ration of autoclaved milk. A gain in weight soon became apparent and the scorbutic symptoms disappeared.

An average consumption of 75 cc. raw milk a day was therefore insufficient to protect this animal from scurvy.

Jane. (No. 1, 1A, *Macacus cynomolgus (fasciatus)*. Common macaque. Female. Initial weight 2090 g.) Dried milk, average daily ration equivalent to 175 cc. Scurvy developed and was cured later by an equal ration of raw milk.

This monkey began to show haemorrhage of the gums about the 105th day and leg weakness appeared soon after. About this time the change from primary to secondary dentition took place. On the 96th day one of the upper median incisors was lost and at first the new budding tooth could be seen in the gap; soon however the gum became swollen and purplish and a haematoma developed over the new tooth.

The monkey continued to take her milk well and did not lose weight seriously. The symptoms progressed in the gums which became much swollen and of a dusky purple colour; the haematoma continued unchanged and the new tooth remained uncut. The legs became very weak and almost useless.

On the 142nd day the ration was changed to one of fresh milk and in order to make the comparison perfectly fair, the quantities given were exactly the same in inverse order as had been the quantities of dried milk consumed. Improvement was rapid. In ten days the animal was much more active and weight was put on speedily; in 20 days the haematoma was almost gone and the cutting of the tooth, delayed for over nine weeks by the attack of scurvy, proceeded normally.

After the cure had proceeded for 54 days and was deemed to be complete, the ration of fresh milk was cut down to 125 cc. daily. Good health and fair growth were maintained but on the 68th day of this treatment, it was decided to raise the ration to 200 cc. by adding 75 cc. of autoclaved milk. In this way it was intended to increase the ration of the fat-soluble A growth factor, while keeping the supply of the anti-scorbutic material the same. This change seemed to lend a slight impetus to growth but it seems probable that the monkey was in any case a slow growing one.

After 162 days the animal was still in good health and it was concluded that in this case 125 cc. daily of raw milk provided sufficient of the anti-scorbutic factor, while 175 cc. of dried milk had failed to do so.

Imp. (No. 6, *Cercopithecus sabaesus*. Male. Initial weight 1940 g.) Raw milk ration 125 cc. Protection from scurvy.

This monkey received 125 cc. raw milk as its sole source of the anti-scorbutic factor for 182 days and showed no sign of scurvy throughout.

On the 60th day 75 cc. autoclaved milk was added as the animal, although in excellent condition, was occasionally subject to convulsions. It was thought that these might be of rachitic origin and it was decided to give an additional supply of fat-soluble A in this manner. The convulsions ceased but the rate of growth was never rapid, 125 cc. raw milk daily however seemed to supply enough of the anti-scorbutic factor as no symptom of scurvy was ever observed.

Chirgwin. (No. 7, *Cercopithecus sabaesus*. Female. Initial weight 2770 g.) Dried milk ration 200 cc. for short period, followed by raw milk ration 150 cc. Result inconclusive.

This monkey started under somewhat unsatisfactory conditions and never cooperated well at any time. It was originally intended that she should receive a ration of 200 cc. of dried milk which she took more or less regularly for 40 days; from that period she refused to touch it and the experiment had to be altered to one with a daily ration of 150 cc. raw milk. This amount was regularly consumed, for any lapses were made up by proportional increase on succeeding days.

Growth was very slow but the animal refused to drink an additional 50 cc. of autoclaved milk which was offered in order to raise the total volume to 200 cc. and to afford an increased supply of fat-soluble growth factor. On the 104th day of the raw milk experiment a slight redness of the gums was noticed, which increased. On the 120th day a large fluctuating swelling on the crown of the head, which had been noticed for several weeks, had become so large that it was decided to operate. No pus was found, only liquid blood which was drained away and the incision sewn up. Such a haematoma was probably not formed traumatically, on account of its slow growth, and it is concluded that the haemorrhage was due to scurvy, a conclusion confirmed by the slightly scorbutic condition of the gums. Contrary to expectation the wound did not fill up again with blood, but healed satisfactorily and at the same time the gums also regained their normal healthy appearance.

The opening of the haematoma took place on May 17, 1919, and on enquiry it was ascertained that May 13th was the date upon which the herd, whence the fresh milk was derived, had been turned out to pasture. The conclusion drawn, therefore, is that the alteration in diet of the cows had raised the anti-scorbutic value of the milk from a ration below, to a ration just above, the protective minimum. The preliminary feeding on dried milk had ended 15 weeks before any scorbutic symptoms began to show, this may have had an influence upon the subsequent history, but it seems unlikely.

The ration of 150 cc. raw milk was continued till the 188th day when the experiment was terminated; the animal seemed in excellent health, there were a few minute bleeding points on the gums, however, which may have been of scorbutic origin.

The conclusion to be drawn from this experiment seems to be that 150 cc. raw milk was in this case just on the borderline of protection, the discrepancy between this result and that of the two preceding experiments, where Jane and Imp were protected with 125 cc. raw milk daily, is doubtless due to individual idiosyncrasy and also possibly to differences in the size of the animals, for the animal at present under consideration was considerably larger than either of the other two.

Caesar. (No. 3, *Cercopithecus callitrichus*. Male. Initial weight 2740 g.) Dried milk equivalent to 250 cc. Result inconclusive.

This animal was fed a ration of dried milk equivalent to 250 cc. daily: growth was fairly good until the 88th day, when it ceased. About the 113th day, the animal's activity became

diminished and very slight redness of the gums was apparent. These symptoms continued and the animal became progressively less active with slightly falling weight until the 130th day, when the weight began to rise again, activity was slowly restored and the slight gum symptoms disappeared.

Such a mild attack of scurvy and spontaneous cure without any intentional alteration in the diet or the amount of it taken, is probably to be explained in the same way as with Chirgwin, the preceding monkey. In that case it was attributed to alteration in the properties of the raw milk ration, consequent upon the taking of the cows out to pasture with corresponding alteration in the antiscorbutic properties of their diet and of the milk given by them. In Caesar's case the date of his sickening and recovery was early in June 1919, but as the dried milk used was two to three weeks old, it would probably have been milked in the beginning or middle of May, *i.e.* just about the time that the cows supplying our fresh milk were proved to have changed their habits and diet for the summer. The manufacturers of the dried milk were applied to for information concerning the herd from whose milk the dried milk was prepared, but they were unable to make any definite statement with regard to dates, since the dried milk was prepared from material furnished by a great number of farms. It is, however, a fair assumption that the majority of cows would go to pasture early in May.

It may be concluded from the results of this experiment, therefore, that 250 cc. dried milk, freshly prepared, is about the minimal protective ration for a monkey weighing from two to three kgs.

Rags. (No. 8, *Cercocebus fuliginosus*. Male. Sooty Mangabey. Initial weight 2230 g.) Fresh milk, daily ration 200 cc. Excellent health and growth.

This animal and the next, Toby, form an excellent contrast as they were similar in every particular, save only for the difference in the daily ration of milk fed to them. Their weight curves are set out for comparison in Fig. 3, p. 321.

Rags received 200 cc. raw milk daily for 225 days during the whole of which time he remained in perfect health and added 50 % to his weight. No trace of any symptom of scurvy was ever observed. At the close of the experiment he weighed 3000 g. but notwithstanding his size, 200 cc. daily was a ration ample to protect from scurvy and to promote good growth.

Toby. (No. 2, *Cercocebus fuliginosus*. Male. Sooty Mangabey. Initial weight 2470 g.) Dried milk, daily ration equivalent to 200 cc. Severe scurvy developed, and was cured upon an equivalent ration of fresh milk, scalded.

This animal was fed 200 cc. daily, and thrived and grew well for about 70 days (Fig. 3, Curve A). After the 70th day growth slackened and it was noticed that there was a slight diminution in the animal's agility. By the 77th day, the symptoms were definite, the gums were swollen and purple and the hind limbs showed distinct weakness. The symptoms still progressed and the monkey lost flesh rapidly. On the 97th day he could not walk at all but was only able to swing himself in a sitting posture and his gums bled freely. On this day it was decided to attempt a cure by increasing the daily ration to 300 cc. of dried milk. The amount was taken readily and continued for a week, but the weight still fell and the general condition became worse.

On the 104th day the ration was changed to 200 cc. of fresh milk which had been "scalded" (see p. 317). The day after this treatment was commenced a slight improvement was detected, in four days the animal could scramble out of his cage and in a week was markedly better. The fall in weight was, however, not arrested for six days and a definite rise in weight did not set in for over a fortnight, when the monkey began to gain in weight with astonishing rapidity. In 16 days after the cure began, he could run about; in 62 days after, he was deemed to be completely recovered and the experiment was ended. Dried milk equivalent to 200 cc. therefore was insufficient, and 200 cc. raw milk, scalded, was amply sufficient to protect this animal from scurvy.

APPLICATION OF THE FOREGOING RESULTS TO THE FEEDING OF INFANTS.

At the present time dried milk is being used for the feeding of infants to an ever increasing extent and it cannot be denied that it offers many advantages over ordinary raw milk both in general convenience and in absence of risk from tuberculosis and other infections. The question arises as to whether its nutritive properties are equal to those of raw fresh milk, or boiled fresh milk, and, seeing that its anti-scorbutic value has definitely been proved to be lower, whether this defect is of serious importance, when it is employed for the nourishment of infants.

Many infant specialists having large experience of feeding with dried milk declare that they have never encountered a case of scurvy among the children fed upon it [see Lane Claypon 1916]. It is not, however, stated always for how many months an exclusive diet of dried milk was continued nor whether all extra anti-scorbutics were excluded. It must also be remembered that slight scorbutic defects may exist without giving rise to the classic appearance of severe infantile scurvy and may even escape detection altogether. Such conditions have been observed by Hess and Fish [1914] and by Miller [1917]. But in absence even of such mild symptoms as were described by these workers, there might yet be conditions which were unsatisfactory as regards the health of the infant, due to an insufficient supply of the anti-scorbutic material in the diet. The experimental work upon scurvy has shown that there may be defects in the structure of bony tissues, only discoverable by microscopical examination, when there has been only a slight deficiency of anti-scorbutic material in the diet, and when no macroscopic signs of scurvy have been detected during life or post-mortem [see Delf and Tozer 1918; Zilva and Wells 1919]. For example, it is during the first months of an infant's life that processes are at work upon the construction of the permanent teeth, and any defect in the structure of these may not be apparent until many years later. It is probable, therefore, that an entirely trustworthy estimate of the anti-scorbutic value of dried milk as the sole food during infancy cannot be formed until this point has been investigated.

The results of the experimental work leave no doubt that dried cow's milk is inferior to raw milk in anti-scorbutic properties and that raw cow's milk itself is a food of comparatively poor value in this respect. It is reasonable to assume that in a diet of raw cow's milk there is not any great excess of anti-scurvy vitamine above the infant's requirements and that when dried milk is substituted, there is risk that the supply of this vitamine may fall below what is necessary for complete safety. Under these circumstances the only wise course is to provide a supplementary anti-scorbutic.

With regard to the growth promoting properties of milk, and in particular its content of the fat-soluble accessory factor, no evidence was obtained from the present work, either from the experiments with guinea-pigs or from those with monkeys, that any loss is suffered during the process of drying. The

results of a separate series of experiments upon this point by one of us (R.E.B.) are shortly to be published. The results of this work also go to show that the growth promoting value of fresh milk and dried milk are roughly about equal. If, as seems probable, from the work of E. Mellanby [1918, 1919], the fat-soluble growth factor and the anti-rachitic factor prove to be the same, then whole dried milk can be relied upon to possess the same value as fresh milk for maintenance of growth and for prevention of rickets. As regards the water-soluble B accessory growth factor, its presence in abundance in many dry foodstuffs indicates that dried milk may be assumed to possess it in about the same amount as fresh milk. As a matter of fact none of the foregoing experiments was designed to give information upon this point, as the water-soluble or anti-neuritic factor was provided also in the grain ration of the experimental animals. The question is of importance, however, when, as is the case with infants, the diet is composed exclusively of milk.

There is urgent need for experimental work in which comparison is made between human milk and cow's milk in respect of the content of the three accessory factors, (1) anti-scorbutic, (2) fat-soluble (or anti-rachitic), (3) water-soluble or anti-neuritic factor. There is at the present time no direct information upon this point and such results would be of great value in helping to settle many of the problems of infant feeding. The obstacle to such work is the great difficulty in obtaining a regular supply of human milk for the long period of time required for trustworthy experiments of this type.

On theoretical grounds we should expect the milk of pasture-fed cows to be richer in the first two of the above vitamins, seeing that a diet of green leaves is a rich source of both. Moreover cow's milk is constituted to be the sole nourishment of the calf, an animal of much quicker growth than the human infant, and therefore presumably needing a more abundant provision of all diet constituents, including the vitamins. On such a basis of argument summer milk from cows may be supposed to contain an amount of accessory factors in excess of the requirements of the human baby. On the other hand, it must be remembered that human milk has important advantages in that it is always taken in the raw condition and undiluted, and that the above arguments in favour of cow's milk refer only to the milk given on the natural diet of grass. In the winter stall-fed cows commonly receive an artificial diet of hay, roots and oil-cake prepared from seeds, which is a diet deficient in the fat-soluble growth factor and, especially if the roots consist of mangolds and not of swedes or turnips [see Chick and Rhodes 1918], deficient also in the anti-scorbutic factor. The milk given will show corresponding deficiencies.

Seeing therefore that cow's milk is a foodstuff of inconstant value as regards its anti-scorbutic properties and that the latter are much reduced in the process of drying, there is every reason to advocate the use of an extra anti-scorbutic in the dietary of infants nourished on dried milk. The results of experiments specially carried out to determine the most suitable substances

for this purpose have been published by Chick and Rhodes [1918], working in this Institute, and among the foodstuffs investigated, raw orange juice and raw swede juice were specially recommended both for potency and for general acceptability. Hess and Unger [1918] have recommended the use of the juice of raw or tinned tomatoes, and their results, as regards tinned tomatoes, have since been confirmed by Chick and Rhodes [unpublished experiments].

SUMMARY AND CONCLUSIONS.

1. A series of nutritional experiments with guinea-pigs is described in which the diet consisted of oats and bran, and a ration of cow's milk constituted the only anti-scorbutic material. It was found possible to prevent scurvy and to maintain good health in animals of 300 to 400 g. weight if 100 to 150 cc. of raw cow's milk were consumed daily. When dried milk was substituted for the fresh milk scurvy was not prevented even when amounts equivalent to these were taken. This was true whether the dried milk was of recent manufacture (one to two weeks old) or of much greater age (six to twelve months).

2. A similar result was obtained in a series of experiments with monkeys. In this case only dried milk of recent manufacture was used. Rations of fresh milk of 50 to 75 cc. daily were not sufficient to prevent scurvy in young animals of 2 to 3 kg. weight; a ration of 100 to 150 cc. daily was however adequate. In case of dried milk the equivalent of about 250 to 300 cc. (at least) was necessary. In one instance severe scurvy, developing on an average daily ration equivalent to 175 cc., was cured rapidly and completely by substituting an equal ration of raw milk. In another case, where scurvy developed on an average daily ration of dried milk equivalent to 200 cc., increase of the ration to the equivalent of 300 cc. caused no improvement, but a cure was successfully effected with a daily ration of 200 cc. "scalded" milk, *i.e.* raw milk quickly brought to the boil and immediately set to cool (exposure to a temperature between 70° and 100° for about five-and-a-half minutes).

3. No indication was obtained from either series of experiments that the growth promoting properties of fresh milk suffer any serious damage during the process of drying.

4. Evidence is brought forward indicating that summer cow's milk has a higher anti-scorbutic value than that given in the winter. This is to be explained by the difference in diet, from fresh herbage in the summer, to hay, oil-cake, cereals and roots in the winter. The use of turnips or swedes, rather than mangolds, should result in a milk of higher anti-scorbutic value.

5. The bearing of these results upon infant feeding is discussed and arguments are given in favour of employing an extra anti-scorbutic in the diet of infants nourished upon dried cow's milk. For this purpose the raw juices of oranges, swedes, and tomatoes are specially to be recommended and in case

of the last named, good results have been obtained even with the tinned material.

The above experiments were carried out under the guidance of Dr Harriette Chick in continuance of work already published in collaboration with her and others, and the authors wish to express their gratitude to her for her help and advice throughout. Thanks are also due to Mr Willis Ginger of the Glaxo Milk Co. and to Mr Wilfred Buckley for kindly furnishing information with regard to the materials used for the experiments, also to Mrs E. Price for timely help in the feeding experiments, to Miss Tozer and Miss Rhodes and lastly to Miss Mary Tazelaar, for her devoted ministrations to the monkeys.

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XXXIII. ON THE MECHANISM OF OXALIC ACID FORMATION BY *ASPERGILLUS NIGER*.

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REPORT TO THE MEDICAL RESEARCH COMMITTEE.

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THE production of organic acids, as a result of the growth of micro-organisms in solutions containing sugars, is of great importance both from a theoretical and practical point of view. A comparison of the acids produced by different organisms reveals the fact that, whereas the *bacteria* as a class chiefly produce monobasic acids, *e.g.*, acetic acid, butyric acid, and lactic acid, the formation of polybasic acids, *e.g.*, oxalic acid, citric acid, and fumaric acid, is almost entirely limited to the *fungi*. Even of the fungi the only family known to possess this chemical activity is that of the Aspergillaceæ. Although there were previously in the literature a number of isolated references to the production of oxalic acid by *Aspergillus niger*, the proof that this micro-organism really forms oxalic acid from sugar was not satisfactorily established until Wehmer published his classical researches on the subject in a series of papers in 1891. In addition to investigating the production of free oxalic acid from glucose under a variety of different conditions, he also showed that salts of oxalic acid are produced by the same fungus from salts of a few organic acids—notably from tartrates, malates, and citrates. He made no attempt, however, to offer any chemical explanation of the steps involved in the process. More recently Emmerling [1903] investigated the production of oxalates, by *A. niger*, from amino-acids and other protein derivatives. In particular, although he found that ammonium oxalate was produced in good yield from aspartic acid, he was quite unable to obtain any from the chemically allied substances—the ammonium salts of succinic, malic and tartaric acids. Heinze [1903], in a paper singularly devoid of experimental details, states that in the decomposition of glucose by *A. niger*, acetic acid is produced in addition to oxalic acid.

Until recently, it has been generally accepted that the production of citric acid from sugar was characteristic of a group of fungi, to which Wehmer gave the generic name of Citromyces, and that oxalic acid fermentation was almost equally characteristic of *Aspergillus niger*. That this is not so was proved by Currie [1917]. Working with a number of different strains of *A. niger*, he

showed that all of them produced from saccharose both oxalic acid and citric acid, the proportions of which could be varied by varying the conditions of the experiment. He pictured the general equation of metabolism of *A. niger* as follows:

Carbohydrate \rightarrow citric acid \rightarrow oxalic acid \rightarrow carbon dioxide \rightarrow mycelium,

and says "The equation has been written in this order, because *Aspergillus niger* will readily form oxalic acid when given only citric acid as a source of carbon."

A short time ago, Wehmer [1918] isolated another member of the *Aspergillaceæ*, to which he gave the name *Aspergillus fumaricus*, which produces from sugar fumaric acid, together with a little citric acid. These three polybasic acids, oxalic, citric, and fumaric, are not to be regarded as extraneous by-products of the degradation of sugar by these micro-organisms, as is, for example, the succinic acid produced during the alcoholic fermentation of sugar, which has been shown by Ehrlich [1909] to have its origin in the glutamic acid of the yeast protein. A consideration of the yield, which is in each case above 50 % of the sugar consumed, shows that these acids really represent definite stages in the degradation of the sugar molecule.

It will be noticed that, with the exception of Currie, no worker has attempted to offer any explanation as to the steps involved in the formation of these acids from sugar. The experiments in this paper were undertaken in the hope of elucidating the mechanism of the production of oxalic acid from sugar by *Aspergillus niger*. It is proposed to study the production of citric acid at some future time.

From a consideration of the literature, we came to the conclusion that a direct method of investigation, though admittedly more satisfactory, offered less hope of success than an indirect attack. The former method involves the isolation of intermediate products, in this case between sugar and oxalic acid, since it is certain that this action does not take place in one stage. The latter method involves the investigation of the production, or non-production, of oxalic acid from possible intermediate compounds, on the assumption that a failure to produce oxalic acid from the substance under investigation justifies the conclusion that that substance does not represent a stage in the reactions involved in the production of oxalic acid. Production of oxalic acid, however, does not necessarily indicate that the substance in question is an intermediate product.

For these reasons, the method employed was to estimate the oxalic acid formed (but present as a salt of oxalic acid) when *A. niger* was cultivated in solutions containing salts of organic acids as the sole source of carbon. We were led to use salts of organic acids, rather than the free acids themselves, since, according to Wehmer, free oxalic acid, in the absence of other available carbon, is readily decomposed by *A. niger* at 37°, whereas fixation of the acid, as a salt, prevents its further decomposition by the fungus to any appreciable extent.

EXPERIMENTAL.

Throughout this work one strain of *Aspergillus niger* was used. This was obtained from Dr Paine of the Imperial College of Science and Technology, S. Kensington, and was identified by Mr F. T. Brooks of Cambridge. To both of them our thanks are due.

A culture solution was made up with the following composition:

Water	1000 cc.
NH ₄ NO ₃	25 g.
KH ₂ PO ₄	12.5 g.
MgSO ₄ ·7H ₂ O	6.25 g.

20 cc. of this, diluted to 50 cc., gave one of Wehmer's solutions, having the following composition:

Water	50 cc.
NH ₄ NO ₃	0.5 g.
KH ₂ PO ₄	0.25 g.
MgSO ₄ ·7H ₂ O	0.125 g.

This solution is subsequently referred to as "Culture Solution A." In some cases 1.5 g. of a salt of the acid under investigation was weighed out, dissolved in 30 cc. water, and to this 20 cc. of the undiluted culture solution was added. In other cases, where it was more convenient to use the free acid as a starting point, 1.5 g. of the acid was weighed out and neutralised with the necessary amount of normal alkali (*N* soda or *N* ammonia), as determined by titration. The volume was then made up to 30 cc. by adding the calculated amount of water from a burette, and, as before, 20 cc. of the culture solution was added. In each case the 50 cc. of solution was contained in a 200 cc. flask, autoclaved, inoculated with spores of *A. niger*, and incubated at 37° in the dark.

After a definite period of time, the oxalic acid content was estimated quantitatively by the usual method. The fungus was filtered off, and thoroughly extracted with hot dilute HCl, and hot water. The combined filtrate and washings were made distinctly alkaline with ammonia, and then acid with acetic acid. The oxalic acid was then precipitated from the boiling solution as the calcium salt, by the addition of calcium acetate and acetic acid. After standing overnight, the washed calcium oxalate was titrated with standard permanganate in the usual way.

This method was shown to be quantitative by adding a known weight of oxalic acid to 50 cc. of the culture solution A, and estimating as above.

	Oxalic acid added to culture solution	Oxalic acid estimated
(a)	0.1964 g.	0.1967 g.
(b)	0.1954 g.	0.1944 g.

The only difficulty met was with culture solutions containing tartaric acid. On standing overnight, a crystalline precipitate, probably consisting of calcium racemate, was found to be mixed with the calcium oxalate. The mixed calcium salts were filtered off, washed with hot water, re-dissolved in hot *N* HCl, made

alkaline with ammonia, and then made acid with acetic acid. After the addition of a little calcium acetate, and while the solution was still hot, the calcium oxalate was filtered off, washed, and estimated in the usual way. The calcium racemate remains in solution, and though a very small amount of calcium oxalate may also remain dissolved in the hot solution, the method is sufficiently accurate for our purpose.

Reference to the results given later shows that we were unable, under any conditions, to obtain any oxalate formation from salts of lactic acid. It was thought possible that the method might not be strictly quantitative in the presence of lactic acid. That this is not so was proved in the following way. To the filtrate and washings from sodium lactate C (Table IV, p. 335), to which had been added calcium acetate in the usual way, without any precipitation of calcium oxalate, was added 100 cc. standard oxalic acid solution (= 15.20 cc. potassium permanganate solution). The precipitated calcium oxalate required 15.46 cc. permanganate solution.

Preliminary Experiments.

In order to obtain some idea of the activity of *A. niger*, the amount of oxalic acid produced from the ammonium salts of a series of acids was determined. (In the case of glyoxylic acid, the calcium salt was used.) The results are summarised in the following table:

Table I.

1.5 g. acid as ammonium salt. Culture Solution A.

Acid				Incubation period in days	Growth	Oxalic acid produced, expressed as g. $C_2H_2O_4$	Percentage of theoretical yield
Butyric	27	—	—	—
Succinic	24	+++	0.3764	16.45
Fumaric	25	+++	0.2671	11.48
Malic	25	+++	0.3477	17.26
Tartaric	26	++	0.0609	3.39
Propionic	27	—	—	—
Pyruvic	26	+++	0.0115	0.53
Lactic	26	++++	0	0
Acetic	26	+	0.0201	0.93
Glycollic	26	+	0	0
Glyoxylic (Ca salt)	32	+	0	0
Culture solution A as control				32	trace	0	0

++++ indicates extremely heavy growth.

+++ „ heavy growth.

++ „ fairly good growth.

+ „ distinct growth.

— „ no growth.

The oxalic acid produced is calculated as anhydrous $C_2H_2O_4$. The theoretical yield is calculated on the assumption that all the carbon of the acid

investigated (determined by titration) is converted into oxalic acid, and our results are expressed as a percentage of this value.

These results indicate that the chemical constitution of the acids, given as a source of carbon to the fungus, is of primary importance for the production of oxalic acid. Thus, although the best growth was obtained with the three-carbon acids, pyruvic and lactic, the amount of oxalic acid produced was nil or negligible, whereas with the four-carbon dibasic acids—succinic, fumaric, malic and tartaric—good yields were obtained. Similarly with the two-carbon acids—acetic, glycollic and glyoxylic—although growth was approximately the same in each case, acetic acid was the only one from which any oxalic acid was produced. It will be noticed that, although *A. niger* grew slightly in the culture solution alone, there was no growth at all when the ammonium salt of propionic acid or butyric acid was present. This fact was also observed by Wehmer, who was unable to obtain any growth from these two acids, under any conditions. This was probably due to hydrolytic dissociation of the organic salts, with the consequent formation of a certain amount of the free acids, which, above a certain concentration, are known to have a deleterious effect on the fungus [Duclaux, 1889]. For this reason, in the subsequent work two other culture solutions were made up, containing, in place of KH_2PO_4 , the one Na_2HPO_4 , and the other Na_3PO_4 . It was hoped that this would have a beneficial effect in fixing any oxalic acid that might be formed.

Main Series of Experiments.

In the following series of experiments, the conditions of which are similar to those of the preliminary series, three culture solutions were used:

1. *Culture Solution A* has already been described.
2. *Culture Solution B.* A stock solution was made such that 20 cc. diluted to 50 cc. (as with A) had the following composition:

Water	50 cc.
NH_4NO_3	0.5 g.
Na_2HPO_4	0.25 g.
K_2SO_4	0.125 g.
MgCl_2	0.005 g.

3. *Culture Solution C.* A stock solution was made such that, on dilution, (as with A and B) it had the following composition:

Water	50 cc.
NH_4NO_3	0.5 g.
Na_3PO_4	0.25 g.
K_2SO_4	0.125 g.
MgCl_2	0.005 g.

The production of oxalic acid (as oxalates) from a large series of acids, present as sodium, ammonium, or calcium salts, was investigated. The results, which are summarised in the following tables, are expressed as in Table I. We have also incorporated with them, for comparison, the results given in Table I.

Table II.

Four-carbon dibasic acids.

Acid	Salt	Medium	Incubation period in days	Growth	Oxalic acid produced, expressed as g. $C_2H_2O_4$	Percentage of theoretical yield
Succinic ...	Na	A	38	+++	1.0503	45.90
		B	38	++	0.8113	35.46
		C	38	+	0.4227	18.47
		NH ₄	24	+++	0.3764	16.45
		A	38	+++	0.6360	27.80
Fumaric ...	Na	A	38	+++	1.050	45.11
		B	38	++	1.074	46.14
		C	38	++	0.7910	34.00
		NH ₄	25	+++	0.2671	11.48
Maleic ...	Na	A	34	+	0	0
		B	34	+	0.0049	0.28
		C	34	+	0.0105	0.62
		A	38	+	0	0
		B	38	+	0.0080	0.48
		C	38	+	0.0333	1.97
Malic ...	Na	A	38	++	0.6493	32.22
		B	38	++	0.5506	27.33
		C	38	+++	0.951	47.2
		NH ₄	25	+++	0.3477	17.26
		A	38	++	0.3170	15.73
Tartaric ...	Na	A	38	+	0	0
		B	38	+	0	0
		C	38	trace	0	0
		NH ₄	26	++	0.0609	3.39

Table III.

Four-carbon monobasic acids.

Acid	Salt	Medium	Incubation period in days	Growth	$C_2H_3O_4$ g.	Percentage of theoretical yield
Butyric ...	Na	A	38	—	—	—
		B	38	+	0.0080	0.33
		C	38	—	—	—
		NH ₄	27	—	—	—
Isobutyric ...	Na	A	38	—	—	—
		B	38	—	—	—
		C	38	—	—	—
β -Hydroxybutyric	Na	A	38	—	—	—
		B	38	—	—	—
		C	38	—	—	—
Hydroxyisobutyric	Na	A	38	—	—	—
		B	38	—	—	—
		C	38	—	—	—

Table IV.

Three-carbon acids.

Acid	Salt	Medium	Incubation period in days	Growth	C ₂ H ₂ O ₄ g.	Percentage of theoretical yield
Lactic ...	Na	A	38	+ + + +	0	0
		B	38	+ + +	0	0
		C	38	+ + -	0	0
		A	7	+ +	0	0
		B	7	- -	0	0
	NH ₄ Ca	C	7	- -	0	0
		A	26	+ - + -	0	0
		A	38	+ - + -	0	0
		B	38	+ + +	0	0
		C	38	- - +	0	0
Pyruvic ...	Na	A	38	- - +	0.0134	0.61
		B	38	+ +	0.1628	7.44
		C	38	+ +	0.1478	6.75
	NH ₄	A	26	+ + +	0.0115	0.53
Glyceric ...	Na	A	31	+ + -	0	0
		B	31	- + -	0.0213	1.81
		C	31	- - -	0.0258	2.19
		A	38	- + -	0.0120	1.02
		B	38	- + -	0.0838	7.11
	NH ₄	C	38	- - -	0.0181	1.54
		A	38	+ + +	0	0
		B	38	+ + +	0	0
		C	38	- +	0	0
Malonic ...	Na	A	38	-	0	0
		B	38	+	0	0
		C	38	+	0	0
	Ca	A	38	-	—	—
		B	38	-	—	—
		C	38	-	—	—
Propionic ...	Na	A	38	-	—	—
		B	38	+ +	0.0825	4.64
		C	38	trace	—	—
	NH ₄	A	27	-	—	—

Table V.

Two-carbon acids.

Acid	Salt	Medium	Incubation period in days	Growth	C ₂ H ₂ O ₄ g.	Percentage of theoretical yield
Acetic ...	Na	A	38	+ + +	0.3869	38.98
		B	38	+ + +	0.4534	45.70
		C	38	+	0.0111	1.12
		A	38	+ + +	0.4211	42.44
		B	38	+ + +	0.4534	45.70
		C	38	trace	—	—
	NH ₄	A	26	-	0.0201	0.93
		A	38	-	0	0
Glycollic ...	Na	B	38	+	0	0
		C	38	+	0	0
		A	38	- +	0.0034	0.20
Glycollic (a different sample of glycollic acid)	Na	B	38	- +	0.0045	0.27
		C	38	- +	0.0036	0.22
		A	26	+	0	0
	NH ₄	A	26	+	0	0
Glyoxylic ...	Ca	A	32	+	0	0

Table VI.

One-carbon acid.

Acid	Salt	Medium	Incubation period in days	Growth	C ₂ H ₂ O ₄ g.	Percentage of theoretical yield
Formic ...	Na	A	38	++	0	0
	..	B	38	++	0	0
	..	C	38	+	0	0

Control cultures containing only culture solution A, B, or C were inoculated with *A. niger* and grown for 38 days at 37°. No oxalic acid was formed.

In each experiment a control flask, containing the salt of the acid under investigation and culture solution, was incubated without inoculation. In no case was any oxalic acid formed.

INFLUENCE OF THE SOURCE OF NITROGEN ON OXALATE FORMATION.

In the literature on oxalic acid formation from sugar by *A. niger*, frequent references are made to the importance of the source of nitrogen supply. Thus Wehmer, in Lafar's handbook [1910], says "A decisive influence is also exerted by the inorganic bodies present, especially the source of nitrogen for the growing fungus, the liberation of acid (oxalic) being absent (in spite of good growth), when ammonium chloride or sulphate is substituted for potassium, calcium, or ammonium nitrate (even in the presence of sugar),—and, in fact, these additions will prevent the formation of acid in cultures that would otherwise acidify at once." This he explains as follows: The assimilation of nitrogen from a nitrate by the fungus results in the liberation of a base from the nitrate, *i.e.*, NH₄OH, NaOH, KOH or Ca(OH)₂, which would fix a portion of the oxalic acid formed, thus preventing its decomposition. The assimilation of nitrogen from ammonium chloride or sulphate, however, would result in the production of a mineral acid—HCl or H₂SO₄—which would tend to favour the decomposition of the oxalic acid, which is known to be much more easily decomposed by *A. niger* than are its salts. This explanation seems a little difficult to accept, in view of the following facts:

1. Only a relatively small amount of the nitrogen supplied is metabolised as protein, as we have proved.
2. Currie [1917] has shown that oxalic acid, along with citric acid, is produced from sugar in culture media made acid to P_H 3.4–3.5 with HCl.

It seemed to us desirable, because of the importance that we attached to acetic acid as a probable precursor of oxalic acid, to prove whether the nitrate ion has any specific importance in the production of oxalates (as distinct from free oxalic acid) from sodium acetate. With this end in view, *A. niger* was cultivated on solutions containing sodium acetate as the sole source of carbon, the nitrogen being supplied as ammonium nitrate, sulphate, chloride or phosphate.

Otherwise the conditions of the experiment were as before. The results given in Table VII are expressed in the same way as in the other tables.

Table VII.

1.5 g. crystalline *Na acetate* (contains 0.904 g. $C_2H_3O_2 Na$).

Ammonium salt	Medium	Incubation period in days	Growth	$C_2H_3O_4$ g.	Percentage of theoretical yield
NH_4NO_3	A	38	+++	0.4211	42.44
	B	38	+++	0.4534	45.70
	C	38	trace	—	—
$(NH_4)H_2PO_4$	A 1	38	++	0.4204	42.37
	B 1	38	+++	0.4836	48.73
	C 1	38	+++	0.4873	49.11
NH_4Cl	A 2	38	+++	0.4067	40.98
	B 2	38	++	0.4739	47.77
	C 2	38	trace	—	—
$(NH_4)_2SO_4$	A 3	38	+++	0.4054	40.86
	B 3	38	++	0.4707	47.43
	C 3	38	trace	—	—

Culture Solutions:

A, B, C have the same composition as previously.

A 1, B 1, C 1 have the same composition as A, B, C, with the exception that NH_4NO_3 is replaced by the same weight of $(NH_4)H_2PO_4$.

A 2, B 2, C 2, NH_4NO_3 replaced by NH_4Cl .

A 3, B 3, C 3, NH_4NO_3 „ „ $(NH_4)_2SO_4$.

These remarkably concordant results show that the nitrate ion has no specific effect on oxalate formation, and confirm the previous observation that *A. niger* can oxidise acetic acid to oxalic acid very smoothly.

DISCUSSION OF RESULTS.

A consideration of the results obtained with the four-carbon dibasic acids shows that, in general, they give remarkably good yields of oxalic acid. In particular, this is especially true of succinic, fumaric, and malic acids. The failure of *A. niger* to produce any oxalic acid from the sodium salts of tartaric acid is probably due to the fact that the fungus only grew very slightly. The poor growth and poor yield of oxalic acid obtained with sodium maleate is in agreement with the observation made by Buchner [1892], who showed that *A. niger* could utilise fumaric acid, but not maleic acid, as a source of carbon. This he was led to expect from the fact that, whilst fumaric acid is a normal constituent of many plants, the isomeric maleic acid has not yet been found in nature. The small amount of growth we did obtain was probably a result of the molecular rearrangement of a portion of the sodium maleate, under the conditions of our experiment.

The outstanding feature of the experiments with the four-carbon monobasic acids is the almost entire absence of growth and failure to produce oxalic acid. The isolated case in which growth and oxalic acid formation was observed justifies the conclusion previously made as to the reason for the failure of the fungus to grow in a solution containing ammonium butyrate.

Most surprising and unexpected results were obtained from the series of acids containing three-carbon atoms. In almost all cases we obtained magnificent growths of the fungus, which were, on the whole, better than those obtained with the four-carbon dibasic acids. In spite of this, the yield of oxalic acid was either nil or very small. In particular was this the case with lactic acid, which is known to be an almost constant product of the bacterial degradation of sugar. A somewhat similar result was obtained by Wehmer [1891]. He cultivated *A. niger* on a solution identical with our culture solution A, and obtained the following results. In one experiment with free lactic acid he obtained no oxalic acid after 101 days; in two experiments with potassium lactate, after the same length of time, he observed the formation of 3.67 % of oxalic acid in the one case, and of no oxalic acid in the other. Finally with calcium lactate, in two experiments having an incubation period of 43 days, he obtained 0.76 % and 0.96 % respectively. That the oxalic acid is not first formed, and then decomposed, is indicated by our results, which proved its non-production after an incubation lasting only seven days.

A comparison of the results obtained with the two-carbon acids—acetic, glycollic, and glyoxylic—shows a sharp differentiation between acetic and the other two acids. With the exception of the one experiment with ammonium acetate, on which the fungus only grew very slightly, the yield of oxalic acid obtained from acetic acid was from 39 % to 49 %, whereas with glycollic and glyoxylic acids the yield was either nil or negligible. The behaviour of *A. niger* towards these three acids offers another example of the difference between biological and chemical reactions, for although it is a relatively simple matter to oxidise glycollic and glyoxylic acids to oxalic acid *in vitro*, it is difficult to obtain oxalic acid from acetic acid. The ease with which *A. niger* oxidises acetic acid seems to us to be a very significant fact.

No oxalic acid is produced from the one-carbon acid—formic acid—although the growth was relatively good. This suggests that oxalic acid formation is not a question of synthesis.

Three facts have been proved conclusively in the experiments described in this paper:

I. Acetic acid is the only one of the three possible two-carbon acids which is oxidised by *A. niger* to oxalic acid.

II. Taken as a class, the three-carbon acids are not oxidised to oxalic acid by the fungus to any appreciable extent.

III. The four-carbon dibasic acids are, as a class, smoothly oxidised to oxalic acid, whilst the four-carbon monobasic acids give no oxalic acid.

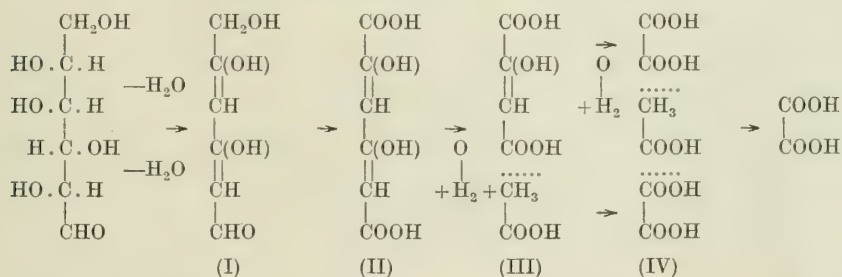
A consideration of these facts leads to the following conclusions:

(I) The breakdown of the sugar molecule by *A. niger* does not take place by a primary splitting of one molecule of sugar into two molecules of a three-carbon acid since, as was pointed out in the introduction, the non-production of oxalic acid from them, by *A. niger*, necessarily precludes the possibility of their being precursors of oxalic acid.

(II) The breakdown cannot take place carbon by carbon, leaving the last two carbon atoms in the chain to be oxidised to oxalic acid, since this would result in the production of one-carbon compounds, and it has been shown that *A. niger* is unable to *synthesise* oxalic acid. Also, if this hypothesis were correct, it would follow that the maximum yield obtainable would be about 33 %, whereas, in practise, the yield is usually about 50 %. It is also extremely unlikely on general grounds.

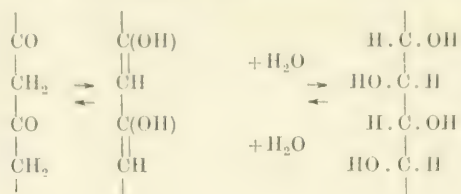
(III) The breakdown probably takes place in two stages, involving in the first stage the production, from one molecule of sugar, of one molecule of a two-carbon compound, and one molecule of a four-carbon compound. This is then broken down in the second stage into two molecules, each containing two carbon atoms.

To meet these conclusions the following scheme is suggested, since it seems to fit in with all the known facts:



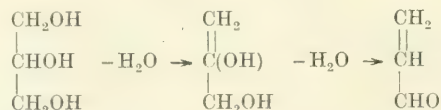
The first stage (I) suggested represents the formation, by simple dehydration, of the enol form of a "polyketide." So long ago as 1893 and, more recently, in 1907, Collie [1893, 1907], who proposed the term "polyketides" for the series of compounds containing $-\text{CH}_2 \cdot \text{CO}-$ groups, pointed out the importance for biological chemistry of this class of compounds. These observations do not seem to us to have received from biochemists the consideration that they deserve. As a class, the "polyketides" give rise to a large series of compounds which either occur in nature or are closely related to naturally occurring compounds. Moreover, the reactions involved are of a very simple nature, *e.g.*, hydration, dehydration, polymerisation, etc., and very often take place at the ordinary temperature. In particular, Collie

pointed out the close relationship between the sugars and the keten group—though at present we have no means of synthesising the sugars from keten or from any of its simple derivatives. Thus, if one takes two keten groups,

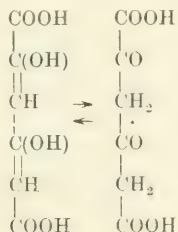


by simple hydration one arrives at a system of four-carbon atoms as present in the sugars.

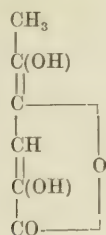
Conversely, the removal of two molecules of water from one molecule of sugar—a change which is probably very easily accomplished by the fungus—would immediately give rise to a very reactive compound. An interesting example of a similar type of change has recently been afforded by Voisenet [1918]. He isolated an organism, to which he gave the name *Bacillus amaracrylus*, which produces acraldehyde from glycerol. The changes involved are represented as follows:



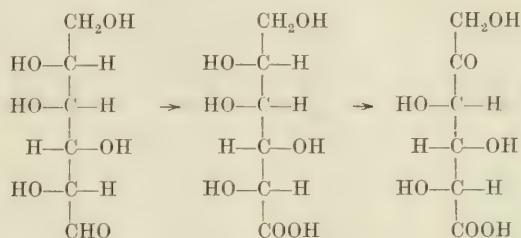
The second stage (II), involving the oxidation of the two terminal carbon atoms to carboxyl groups, is extremely probable in view of the oxidising powers which *A. niger* is known to possess. There is, of course, no evidence to show whether dehydration takes place before oxidation, or *vice versa*, but in either case there would result the formation of the enol form of a diketo-acid:



There are a number of examples of the production, by micro-organisms, of keto-acids from sugars—a fact which renders this change extremely probable. Traetta-Mosca [1914] isolated a fungus having the characteristics of *Aspergillus glaucus* which ferments sugars giving a hitherto unknown substance, which is in all probability the γ -lactone of a trihydroxyhexadienoic acid. For this acid, which is fermentable to alcohol by yeast, Traetta-Mosca suggests the following formula:



Also *Bacterium xylinum* [Adrian Brown, 1886] oxidises glucose first to gluconic acid, and then to keto-gluconic acid:



Weizmann's bacillus [1915], which produces acetone from the carbohydrates of maize, probably produces first aceto-acetic acid, which then loses CO_2 and forms acetone (Author's unpublished observations).

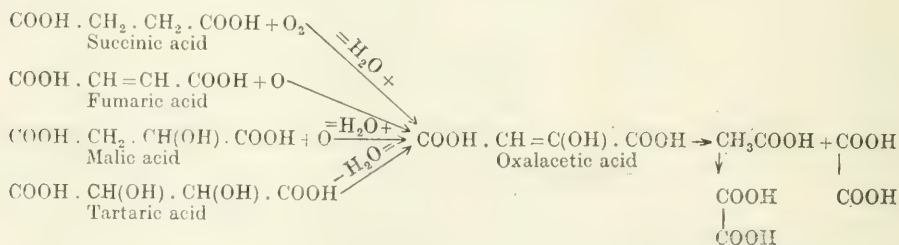
The subsequent breakdown of the diketo-acid II is readily explained. It contains a β -keto-linking, and (disregarding the δ -keto-linking for the moment) is thus a β -keto-acid. The β -keto-acids, as a class, are readily hydrolysed, giving an acid containing two carbon atoms less and acetic acid. This change is represented in stage III. The acetic acid is then oxidised to oxalic acid, a change which our results have shown to be easily accomplished by *A. niger*. The residual part of the molecule III is the enol form of the β -keto-acid—oxalacetic acid—which, undergoing a similar hydrolysis to II, gives rise to one molecule of oxalic acid and one molecule of acetic acid, which is then oxidised, as before, to oxalic acid.

The scheme suggested involves the intermediate formation of acetic acid, and so it might be objected that one ought to be able to identify this acid, as one of the degradation products of sugar by *A. niger*. It must be confessed at once that we have quite failed to do so. Heinze [1903], in a paper previously referred to, states that he obtained acetic acid, in very good yield, along with oxalic acid by the action of *A. niger* on sugar. Unfortunately the lack of experimental details in his paper is so marked—the production of acetic acid being stated as a fact, without any indication as to the method of identification—as to render the statement practically worthless. Obviously the accumulation of acetic acid, and hence the possibility of isolating it, is governed by the relative rates of two reactions:

- (a) The reaction by which it is produced from sugar.
- (b) The reaction by which it is decomposed.

Thus if reaction (a) proceeds more rapidly than (b), acetic acid will accumulate, and one ought to be able to isolate it. If, on the other hand, reaction (b) proceeds more quickly than (a), then it is obviously impossible, at any stage, to isolate any acetic acid. In other words, in order to explain the non-occurrence of acetic acid, one must be able to show that it is decomposed at least as quickly as is glucose. Fortunately there is perfectly satisfactory evidence to prove that this is the case. W. Pfeffer [1895] in a large series of experiments cultivated *A. niger* in solutions containing glucose, together with one of a number of other substances. He was thus able to compare the relative stability of these substances compared with glucose. His conclusions with regard to acetic acid may be given in (a translation of) his own words. "Although a sufficient amount of glucose is quite capable of more or less protecting glycerol or lactic acid from utilisation, this is not true of acetic acid, which is more freely used by the fungus (*A. niger*) than is glucose itself, when simultaneously present."

It was pointed out in the introduction that, although the non-production of oxalic acid from the substance under investigation justifies the conclusion that that substance does not represent an intermediate stage between sugar and oxalic acid, the production of oxalic acid in quantity from the substance under investigation does not necessarily prove that that substance represents an intermediate stage between sugar and oxalic acid. Nevertheless, such a production of oxalic acid justifies the conclusion that the substance in question probably gives rise, in its decomposition, to a substance which is intermediate between these two compounds. Thus, although succinic, fumaric, malic, and, to a less extent, tartaric acid give rise to good yields of oxalic acid, it is not claimed that each of these substances represents an intermediate stage in the degradation of the sugar molecule. If, however, the scheme suggested is accepted, the production of oxalic acid from these dibasic acids is readily explained. Thus, by a simple reaction, the enol form of oxalacetic acid may be supposed to arise from each acid—and hence oxalic acid:



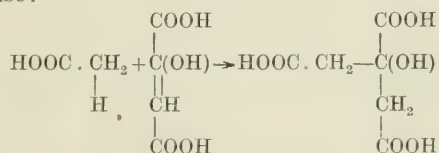
There is experimental evidence, in at least two of these cases, that this change takes place. Wohl and Oesterlin [1901] showed that, by reactions taking place at ordinary temperatures, tartaric acid may be converted into oxalacetic acid. Fenton and Jones [1900] obtained oxalacetic acid from malic acid by oxidation with H_2O_2 and a ferrous salt in the cold.

The principles involved in the suggested scheme also offer an explanation

of the non-production of oxalic acid from the three-carbon acids. The production of a β -ketonic acid, giving rise by hydrolysis to acetic acid, and hence to oxalic acid, is the most essential part of the scheme. If one applies this suggestion to the three-carbon acids, it is seen that no β -ketonic acid, and hence no oxalic acid, can arise from them, since they only contain three carbon atoms. The occasional production of small amounts of oxalic acid from some of them—more particularly from sodium pyruvate in culture media B and C, which were alkaline in reaction—probably can be explained by the fact that secondary changes take place, during sterilisation, which give rise to small amounts of acetic acid.

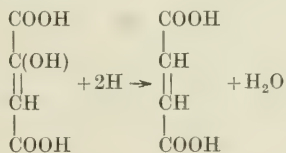
In conclusion, we may be allowed to point out that the scheme suggested for the breakdown of sugar by *A. niger* offers what may be a simple explanation of the production of citric acid by this fungus, and of fumaric acid by *A. fumigatus*.

In stage III of the reactions involved, we have produced one molecule of oxalacetic acid, and one molecule of acetic acid. By the simple union of these two compounds—both of which contain “keten” linkages, which as Collie pointed out readily undergo condensation with one another—a molecule of citric acid would arise:



Citric acid has indeed been synthesised by Lawrence [1897], by condensing oxalacetic ester with bromacetic ester in the presence of zinc. It seems inevitable, in order to explain the production of citric acid from sugar, to presuppose a primary splitting of the sugar molecule, followed by a condensation, because of the presence of a side chain in the citric acid molecule.

The production of fumaric acid might also be easily explained by the reduction of oxalacetic acid:



SUMMARY.

1. *Aspergillus niger* was cultivated on synthetic media containing, as the sole source of carbon, the salts (Na, (NH₄) or Ca) of various organic acids, and the amount of oxalic acid produced was estimated.

2. The following results were obtained:

(a) The four-carbon dibasic acids (succinic, fumaric, malic, tartaric) gave good growth, and good yields of oxalic acid.

(b) With the four-carbon monobasic acids (butyric, isobutyric, β -hydroxybutyric, hydroxyisobutyric) there was almost no growth, and no production of oxalic acid.

(c) The three-carbon acids (lactic, pyruvic, glyceric, malonic, propionic) showed, as a whole, remarkably good growth, but either entire absence, or very small yields, of oxalic acid.

(d) Of the two-carbon acids, acetic acid gave good growth, and good yields of oxalic acid. Glycollic and glyoxylic acids gave fairly good growth, but no oxalic acid.

(e) The one-carbon acid, formic acid, gave fairly good growth, but no oxalic acid.

3. From a consideration of the results obtained, a theoretical scheme is suggested to represent the breakdown of sugar to oxalic acid, involving the intermediate formation of β . δ -diketo-adipic acid. This undergoes hydrolysis into acetic acid and oxalacetic acid, which on further hydrolysis gives acetic acid and oxalic acid. The acetic acid, which is produced in each case, is then itself oxidised to oxalic acid. It is also suggested that the formation of citric and fumaric acids from sugar, by the *Aspergillaceæ*, may be referred to the intermediate production of oxalacetic acid.

We take this opportunity of thanking Professor F. Gowland Hopkins most sincerely for his kind encouragement and criticism during the progress of this work.

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XXXIV. ON THE SELF-PURIFICATION OF RIVERS AND STREAMS.

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It is well known that the purifying action of river-water polluted with sewage is very considerable, as a few miles below the outfall or point of pollution a river may show little or no sign of pollution at all. Purification is effected by sedimentation of the suspended solids, and oxidation of the soluble material. The processes of oxidation give rise to deoxygenation of the river-water, and the extent of deoxygenation depends on the strength of the sewage, the degree of dilution afforded by admixture with the river-water, and the velocity of the river.

If the concentration of oxidisable material be excessive, the river-water will suffer considerable or complete deoxygenation, and a nuisance will result owing to the septic condition caused by the anaerobic decomposition of the organic matter. On the other hand, if there be sufficient dilution, the organic matter can be oxidised and thus destroyed without depriving the river-water of oxygen to any appreciable degree. The suspended matter will also be sedimented in the form of a thin film distributed over a considerable area of river-bed, and no nuisance will thus result through the formation of foul mud-banks.

Recovery from pollution, or self-purification, as it is termed, thus depends on the conditions obtaining with the particular river. Ordinarily towns situated on the same river are sufficiently separated to give time for the river to recover from the effects of the upper pollution before it is subjected to the next. On the other hand, if towns be close together, a nuisance may result, and the river may become unfit to receive a further volume of sewage lower down, until a considerable length of time and dilution from tributaries enable purification to be effected.

As early as 1891 Pettenkofer [see *Roy. Comm.* 1913, p. 65] appreciated the fact that rivers are capable of self-purification and stated that the condition of a river below the point of pollution could be roughly foretold provided the population of the town and the volume of the river were known. He also realised that dissolved oxygen in the river-water played an important part in the process of self-purification.

A certain amount of work has been carried out by the Royal Commission on Sewage Disposal to ascertain the rate of self-purification of polluted river-

waters as measured by the change in the amounts of oxidisable carbonaceous matter and ammonia present, and the production of nitrites and nitrates.

In laboratory experiments it was conclusively shown by Adeney [*Roy. Comm.* 1908] that in mixtures of sewage and river-water the oxidation of the carbonaceous matter precedes that of ammonia. In the case of rivers and streams under natural conditions however the disappearance of the ammonia takes place practically *pari-passu* with the oxidation of the carbonaceous matter [*Roy. Comm.* 1913, p. 63]. This disappearance of ammonia is due to a large extent to absorption by algae and aquatic plants [*Roy. Comm.* 1913, p. 85].

It would also appear that nitrification actually takes place in the polluted river-waters, and this is an additional factor bringing about the disappearance of the ammonia [*Roy. Comm.* 1913, p. 89].

The effect of temperature on the rate of oxidation of organic matter, *i.e.* absorption of dissolved oxygen, was also considered by the Commission [1913, p. 94], especially in relation to the question of standardisation of sewage effluents. Observations were made on the temperatures of different river-waters during the year, and the mean maximum temperature was found to be about 65° F. or 18.3° C. The dissolved oxygen absorption by sewage effluents in five days was found to be much greater at temperatures of 22–24° C. than at 18.3° C., and it was accordingly recommended that in the standardisation of effluents by the dissolved oxygen absorption test a definite temperature should be adopted and that this should be 18.3° C.

Seasonal variation is therefore another factor in the self-purification of rivers. It is clear that owing to the increased rate of oxidation of organic matter due to greater bacterial activity, and the removal of ammonia due to plant development, the process of purification will operate more rapidly during the warmer months. On the other hand, if the river-water be over-charged with sewage, the nuisance may be greater in the summer months than in the winter, as the increased rate of oxidation may lead to deoxygenation of the water.

Although the subject of self-purification of rivers has thus been considerably studied, no work so far seems to have been carried out to ascertain whether the rate and course of purification are influenced by the geological source of the river. Some evidence has already been obtained by the authors [1918] that there is a considerable difference in the rates of oxidation of organic matter in tap-waters and river-waters, the difference being so great that it is necessary to employ the river-water into which the effluent is discharged as the diluting medium in the dissolved oxygen absorption test, in order to obtain results of any value in the standardisation of effluents. It is quite possible that the mineralogical constituents of river-waters may exercise specific influences—either by acceleration or retardation of the oxidation processes.

It may also be expected that the particular types of organic matter derived

from the plant and animal life of rivers and from drainage of land, and the numbers and kind of bacteria and other micro-organisms will have important influences. These however ultimately depend to a large extent on the geological source of the river.

The complex chemical and biological processes going on in river-beds or in the supernatant river-water no doubt have some relation to those going on in soil. There are however differences in the conditions controlling these processes in rivers and on land.

First of all, there is frequently much less humus in a river-bed than in the soil, so that in rivers the chemical and biological processes may be said to be actually going on in a geological bed and uncomplicated by large amounts of decomposed vegetable matter.

Furthermore, in a particular soil there is as a general rule a constancy in the amounts and proportions of the mineralogical constituents. In the case of rivers, on the other hand, except at the spring-head there must be at various times considerable variation, as a river may receive contributions of water from many springs and from drainage off large areas of land of different geological formations, and the relative volume of water from these various sources must depend on climatic conditions and seasonal variations. The conditions obtaining are thus extremely complicated.

Notwithstanding these variations, a river-water may be sufficiently characteristic in chemical composition to distinguish it from other river-waters.

For example, in considering the characters of river-waters in this country, we find that they necessarily depend on the nature of the soils and rocks over which they flow. The rivers of Scotland thus are in general soft, the mountains being composed of granite, gneiss, mica schists, and there is only a small proportion of calcium carbonate. The water from the Cumberland and the Welsh mountains (Silurian rocks) is for the same reason generally soft. In Wales however when the Old Red Sandstone district is reached, marls and limestones are found, and the river-waters are harder. Rivers draining areas in which the Carboniferous Limestone, the Permian Rocks, and New Red Sandstone occur, also yield, as a general rule, somewhat hard water, while river-waters derived from the Lias, Oolitic and Cretaceous Rocks are of necessity hard, because Limestone or Chalk as the case may be are so abundant in these formations.

In draining areas of the various geological strata, rivers carry down an enormous amount of mineral and vegetable matter, which is finally discharged into the sea.

For this reason, river-beds, except possibly near the spring-head, are in general "alluvial" in nature, *i.e.* representative of many geological formations. The mineralogical constitution of a river-bed depends to a large extent on the adjacent geological strata and on those drained by the tributaries of the river, and at different times may be thus liable to considerable variation. Even if the actual mineral constituents in certain cases do not vary much, the physical

and mechanical factors, *i.e.* size and form of particles or texture, would vary and these also would be expected to affect the rate of the chemical and biological processes going on in the river-bed, owing to variations in the degree of aeration.

In the Chalk country, for example, the river-beds will tend to consist of fine-grained calcareous loams or marls, in the Lower Greensand formations these will be replaced by sandy loams, often of coarse texture, sometimes also by peaty loams and fine gravel; again, in the case of a stream from the Lower Greensand formations running over the Atherfield Clay, we should expect to find the clay converted into a sandy clay owing to admixture with the sandy detritus from the Hythe beds. Calcareous, glauconitic and cherty matter might also be found, derived from the Bargate beds, Glauconitic Sandstones, and Chert beds present in the Lower Greensand formation.

There are thus reasons for anticipating that the processes of oxidation of organic and possibly inorganic matter may depend on the quality of the river-water into which it is discharged.

The question is not only of interest to geologists, but is also of importance from the point of view of practical sewage disposal, as, if the oxidation of sewage constituents varies perceptibly in different river-waters, it will be necessary in drawing up new plans for sewage purification to consider the nature of the river into which the effluent is to drain before a standard of purification can be selected.

During the last two years therefore many comparisons of the rates of oxidation in different river-waters of the constituents of sewage or sewage-effluents have been made, and the results of this investigation are given in the present paper. The comparisons have been made by determining the dissolved oxygen absorbed by sewage or sewage effluents when diluted with the various river-waters.

Further experiments supplementary to those described in the previous paper [Cooper, 1918] and comparing the rates of oxidation in the presence of distilled water, various tap-waters and river-waters have also been made.

In addition, some questions of technique bearing on the work and also of importance in the standardisation of effluents are discussed.

The general geological aspect of this work has been studied by the late A. E. C. who unfortunately died in the course of the investigation. In addition, he had proposed to study the mineralogical constitution of the river-beds and to correlate the results with those obtained relating to the processes of oxidation, but this part of the work had only been commenced just before his untimely death and is necessarily not far advanced. His help and enthusiasm in scientific work have been greatly missed. The copious notes on the subject left by him have however been carefully read through, and suitable selections have been incorporated in this paper.

The chemical work has been carried out by E. A. C. and J. A. H. at the Hygienic Laboratory, School of Army Sanitation, Aldershot Command.

1. THE ESTIMATION OF DISSOLVED OXYGEN IN RIVER-WATERS AND DILUTED SEWAGE EFFLUENTS.

The method used for the determination of the dissolved oxygen content was that of Winkler, with the Rideal-Stewart modification [*Roy. Comm.* 1913, p. 93].

By this method it was found that aerated distilled water and tap-waters contained 0.93 parts of oxygen per 100,000 at 18.3° C. or 65° F. This is the correct figure according to Roscoe and Lunt's tables [1889].

It was found however that in the case of aerated clean river-waters the oxygen concentration was often somewhat lower, *e.g.* 0.80 to 0.90, while aerated polluted river-waters, and sewage effluents diluted five times with river-water often only contained about 0.80 parts, sometimes, in fact, only 0.70 parts of oxygen per 100,000. As a general rule bad effluents yielded lower results for oxygen concentrations after aeration than good ones, but this was not always the case.

With bad effluents it would not matter whether these low figures were due to experimental error or were realities, as the results would always condemn the effluents. In the case of good or fairly good effluents, the question is of great importance, as is shown by the following examples:

Sewage Effluents.

Initial oxygen concentration after full aeration	Dissolved oxygen absorption in five days at 18.3° C. Parts per 100,000	
	Assuming the oxygen concentrations are correct	Assuming true oxygen concentration is 0.93
1. Effluents 1.5 dilution:		
1. 0.78	0.50	1.15
2. 0.83	0.85	1.25
Canal-water receiving effluent:		
0.91	0.28	0.30
2. Effluents 1.5 dilution:		
1. 0.74	0.20	0.85
2. 0.77	0.60	1.10
Stream-water receiving effluent:		
0.86	0.11	0.18

In the case of good effluents therefore it makes a very considerable difference whether the initial concentration is in reality 0.93 or is the lower figure actually found by experiment.

Both in the routine standardisation of effluents and the carrying out of the investigation on self-purification of rivers it was therefore essential first of all to go into this question of technique.

The low results could be due to at least three factors:

1. The presence of a substance or substances in sewages diminishing the solubility of oxygen in water (in which case the low results would be realities).

2. The presence of oxidisable substances, using up dissolved oxygen or manganese peroxide in the course of the process of estimation.

3. The presence of substances reacting with iodine in the course of the final titration with thiosulphate.

In the latter two cases the actual results obtained for the oxygen concentration are erroneous.

It was thought possible that the sodium chloride present in sewage effluents roughly to the extent of 12 parts chlorine per 100,000 was the cause of the low results. In a sewage effluent diluted 1.5 with stream-water this would yield a chloride concentration of about four parts chlorine per 100,000. Experiments showed however that this concentration of sodium chloride had no effect on the solubility of oxygen in water.

The possible influence of organic matter was then considered. Peptone was dissolved in boiled distilled water just before full aeration and the dissolved oxygen figure of the solution compared with the normal figure at 18.3° C., obtained under the same conditions for distilled water itself. The amount of peptone to be added was judged from the organic nitrogen figure of a fairly bad effluent and amounted to 0.30 g. per litre.

The results obtained were as follows:

Distilled water alone	0.94	0.87	Dissolved oxygen, parts per 100,000
Distilled water + 0.3 g. peptone per litre	0.70	0.70	

It is seen that much lower results were obtained with the peptone solution.

Peptone, and also tank-liquor were next added at various stages of the dissolved oxygen test in order to attempt to ascertain the nature of the interference. The results were as follows:

Dissolved oxygen concentration of boiled distilled water after full aeration at 18.3° C.

1. Distilled water alone (control) ...	0.97	1. Distilled water alone (control) ...	0.92
2. 0.3 g. peptone per litre added before aeration	0.88	2. One-fifth vol. tank-liquor added before aeration	0.85
3. 0.3 g. peptone per litre added immediately after aeration	0.82	3. One-fifth vol. tank-liquor added immediately after aeration	0.83
4. 0.3 g. peptone per litre added before precipitation of the manganese peroxide	0.90	4. One-fifth vol. tank-liquor added before precipitation of manganese peroxide	0.75
5. 0.3 g. peptone per litre added just before the peroxide was dissolved by addition of the HCl	0.93	5. One-fifth vol. tank-liquor added just before solution of peroxide in HCl	0.91
6. 0.3 g. peptone per litre added after addition of the acid	0.95	6. One-fifth vol. tank-liquor added after addition of the acid	0.84
		7. Tank-liquor alone unaerated contained no dissolved oxygen	

It is seen that the organic matter or other constituents of sewage can affect the results at all stages of the process. The low results do not appear to be due to diminished solubility of oxygen, as the organic matter, etc., exerts its influence after aeration and bottling of the water, and even when added

immediately before the precipitation of the oxygen as peroxide of manganese (probably through decomposition of the latter). It can also affect the titration of the iodine with thiosulphate, probably by reacting quickly with some of the iodine.

The latter point is further illustrated by the following additional results:

								Oxygen concentration of distilled water at 18.3° C.
Distilled water alone	0.90
With addition of tank-liquor just before titration of liberated iodine with thiosulphate	0.84

Further experiment also showed that, if a diluted sewage effluent were aerated and allowed to stand for a short time before estimation of the dissolved oxygen, there was also a marked fall in oxygen concentration:

	Immediately after aeration	Standing 40 mins. after aeration
Oxygen concentration	0.86	0.66
" "	0.88	0.79

Such rapid loss of oxygen must probably be due to chemical oxidation of certain substances, *e.g.* ferrous sulphide and organic matter, and points to this factor as one cause of the low results obtained in estimating the dissolved oxygen content of sewage effluents.

The presence of suspended matter in sewage effluents on the other hand seemed to have only a slight effect on the dissolved oxygen figure:

				Parts per 100,000	
Unfiltered	0.84	0.85	
Filtered	0.87	0.84	

From these results it would appear that the low results obtained in estimating the dissolved oxygen content of aerated sewage effluents are due to:

1. Rapid oxidation of certain constituents during the estimation.
2. Decomposition of a portion of the manganese peroxide by organic matter.
3. Loss of iodine in the final thiosulphate titration due to reaction with constituents of sewage.

Attempts were next made to destroy the constituents of effluents causing the interference by (1) doubling the amount, and (2) doubling the time of action of the permanganate used in the Rideal-Stewart modification of Winkler's dissolved oxygen test. The permanganate is primarily used to destroy nitrites, which also interfere with the test, but it was thought that it might also be serviceable in destroying the other interfering substances if sufficient concentration or time were allowed.

Satisfactory results were sometimes but not always obtained when the amount of permanganate was doubled:

Oxygen concentration. Parts per 100,000.

Usual amount of permanganate	Double amount
0.87	0.98
0.88	0.94
0.81	0.81
0.71	0.77
0.70	0.81
0.82	0.82
0.88	0.90
0.83	0.91
0.86	0.83
0.88	0.93
0.88	0.935
0.85	0.90

Doubling the time, *i.e.* allowing 40 mins. instead of 20 mins., also did not give good results:

Usual time	Double time
0.83	0.86
0.85	0.84
0.80	0.91
0.74	0.78

There thus seemed to be no simple method of preventing the low results obtained in estimating the oxygen content of sewage effluents.

Experiments were therefore carried out to ascertain if the error still existed after incubation of the diluted effluent at 18.3° C. as carried out in the Rideal-Stewart or dissolved oxygen absorption test. If the interfering substances were largely oxidised or otherwise destroyed during the incubation, there would obviously be a large error in the initial oxygen estimation, but little or none in the final estimation after five days. In this case, the results for the dissolved oxygen figures of the effluents would be low and a considerable error thus introduced in routine work. On the other hand, if the interfering substances were not appreciably destroyed, the error in estimation would be practically the same at the end of five days as at the commencement of the test, and would thus be neutralised, and sufficiently accurate results would be obtained for ordinary purposes.

Experiments were therefore carried out to throw some light on this matter. Aerated diluted effluents were incubated in closed bottles at 18.3° C. and after five days or other suitable period the contents of the bottles were again *reaerated* strictly at the same temperature and the oxygen concentrations estimated and compared with the initial result.

It is seen that as a general rule there was very little difference in the figures, and, thus, although low results are often obtained in the estimation of oxygen in sewage effluents, the error is neutralised and does not interfere with the utility of the dissolved oxygen test.

Initial oxygen concentration at 18.3° C.	Oxygen concentration after incubation and aeration at 18.3° C.
Parts per 100,000	
0.91	0.90 (5 days)
0.90	0.90 (5 „)
0.87	0.88 (5 „)
0.86	0.85 (5 „)
0.83	0.82 (24 hours)
0.80	0.80 (3 days)
0.80	0.73 (13 „)
0.88	0.82 (24 hours)
0.88	0.86 (7 days)
0.88	0.90 (5 weeks)
0.87	0.86 (5 days)
0.73	0.73 (4 weeks)
0.85	0.94 (4 „)
0.84	0.87 (5 days)
0.80	0.83 (2 „)

2. THE EFFECT OF TEMPERATURE ON THE DISSOLVED OXYGEN ABSORPTION FIGURES OF EFFLUENTS.

Reference has already been made to the experimental work of the Sewage Commission, showing that the oxidation processes were considerably accelerated by rise in temperature from 18.3° to 22° or 24° C., on account of which it was considered essential to carry out the dissolved oxygen absorption test rigidly at 18.3°, the average maximum temperature of rivers in this country.

Some further experiments have been made on the question of the influence of temperature, and are summarised below:

Dissolved oxygen absorption figures for effluents.
Parts per 100,000, five days.

10° C.	18.3° C.	22° C.	37° C.
1.10	1.75	—	—
1.20	2.45	—	—
0.30	0.60	—	—
—	0.65	2.10	—
—	9.80	9.70	9.6
—	11.30	11.10	11.00

(In all these experiments the initial aerations were carried out strictly at the particular temperatures selected.)

It is seen that in one case out of three a very much higher result was obtained at 22° than at 18.3°, while in the two other cases at temperatures of 18.3°, 22°, 37°, no differences were observed. At 10° however the oxidation processes went on very much more slowly than at 18.3°, and the results emphasise the necessity of carrying out the test under strictly standardised conditions. For routine work the standard temperature of 18.3° C. recommended by the Commission has been used.

The figures tabulated above are also of interest from the point of view of the general question of river purification, as they tend to show that sewage will have a considerably less polluting effect in the winter months than during the summer, while it will not necessarily cause a greater pollution in rivers in tropical countries than in temperate regions.

3. THE EFFECT OF TIME ON THE DISSOLVED OXYGEN ABSORPTION TEST.

The period of five days was recommended by the Commission for this test [*Roy. Comm.* 1913, p. 78], as it was suspected that unreliable results were obtained if shorter periods were employed, owing to the possibility of a bacterial lag, and a greater influence due to the presence of inhibitory substances in the sewage. The value of the test, however, would be considerably enhanced, if a shorter period than five days would be sufficient. It was therefore considered advisable to carry out more experiments on this point, and the results are tabulated below:

Dissolved oxygen absorbed at 18.3° C.

	48 hours	5 days	Ratio
1. Effluent	0.14	0.30	1:2
Stream above effluent	0.007	0.014	1:2
Stream below effluent	0.02	0.11	1:5
2. Effluent	0.70	1.00	1:1.4
Stream above	0.04	0.10	1:2.5
3. Primary effluent ...	1.70	3.00	1:1.7
4. Effluent	0.12	0.95	1:8
Stream above	0.12	0.13	1:1
5. Effluent	2.35	All O absorbed	1:?
6. Effluents 1	0.10	0.80	1:8
" 2	0.00	1.45	1:∞
Stream above	0.16	0.29	1:1.8
7. Effluents 1	0.00	0.70	1:∞
" 2	0.18	>3.45	1:>19
" 3	0.16	0.75	1:4.7
" 4	0.46	>3.40	1:>7.4
River above... ..	0.11	0.23	1:2
8. Effluents	0.80	0.65	1:0.8
"	1.20	1.55	1:1.3
Stream	0.08	0.26	1:3

The Commission found that the five days' test gave a very useful idea of the polluting effect of an effluent [*Roy. Comm.* 1913, p. 63], and it was thought that if a fairly definite ratio between the two and five days' tests could be obtained, it would be possible to use the two days' test as a standard. The above results however show that this is not the case, as even very bad effluents may give little or no indication of oxygen absorption in two days.

Further experiments using a stronger concentration of effluent, *i.e.* 1 : 1 dilution with river-water, were next carried out. The results are as follows:

			48 hours	5 days	Ratio
1. Effluent	0.15	1.40	1 : 9
2. "	0.56	0.90	1 : 1.6
Stream above	0.09	0.15	1 : 1.7
3. Effluent 1	0.52	0.65	1 : 1.2
" 2	0.96	1.55	1 : 1.6
Stream	0.08	0.26	1 : 3.2

Even with the increased concentration it is seen there is considerable variation in the ratio, and at the present time there is no alternative but to employ the longer period of five days.

It was thought that it might be possible to accelerate the processes by means of a catalyst, and in this connection the effect of manganese salts has been studied. Small amounts of manganese chloride were added to diluted sewage effluents, the dissolved oxygen contents estimated at the end of two days, and the results compared:

Dissolved oxygen absorbed in 2 days. Parts per 100,000	
Effluent without MnCl_2	0.65
" plus 0.1 % MnCl_2	0.02
" plus 0.025 % MnCl_2	0.03

The addition of the manganese salt thus considerably inhibited the absorption of the dissolved oxygen. Further experiments with other catalytic substances are desirable.

4. THE INFLUENCE OF DILUTION.

The Commission [*Roy. Comm.* 1913, p. 80] carried out some experiments bearing on the question of the effect of dilution on the rate of dissolved oxygen absorption by sewage effluents, and found that dilution up to 1 in 3 with tap-water had very little effect. It was thought desirable to carry out some more experiments, and the results are illustrated by the following figures:

Dilution	Dissolved oxygen absorbed by effluent. Parts per 100,000	
1 in 2	0.52	0.96
1 in 5	0.80	1.20

It is seen that the oxygen absorption is slightly increased by increasing the dilution $2\frac{1}{2}$ times. Increasing the dilution from 1 in 5 to 1 in 20 had practically no influence at all, and dilution would thus seem to be not such an important factor as would be expected.

5. THE POSSIBLE INFLUENCE OF INHIBITION FACTORS.

The possible influence of certain constituents, *e.g.* lime, in sewage in inhibiting the absorption of dissolved oxygen was realised by the Commission [*Roy. Comm.* 1913, p. 84]. It was considered quite likely that the dissolved

oxygen test might for this reason under certain conditions give false and low results, especially if the two days' test was used instead of the five days' test. The possibility of ammonia acting as inhibitory agent does not however appear to be discussed. It has been noticed frequently in routine work that an effluent containing as much as 10 parts per 100,000 of ammoniacal nitrogen might yield a dissolved oxygen absorption figure of only about 1.0 and would thus be classified as a satisfactory effluent. Experiments have been therefore carried out to ascertain whether ammonia in such concentrations has any inhibitory action. Ammonium carbonate was used for this purpose:

Diluting medium 1:5										Dissolved oxygen absorbed by an effluent in 5 days
Distilled water	13.0
"	containing amm. carb.	equivalent to	2.5 parts amm. N	per 100,000						13.4
"	"	"	"	"	5.0	"	"	"	"	13.4
"	"	"	"	"	10.0	"	"	"	"	12.6
"	"	"	"	"	20.0	"	"	"	"	12.2
"	"	"	"	"	45.0	"	"	"	"	11.2

It is seen that ammonia in concentrations such as occur in sewage effluents had no perceptible influence on the rate of oxygen absorption. Much larger concentrations in fact had only a small effect.

6. A COMPARISON OF THE CAPACITIES FOR SELF-PURIFICATION OF DIFFERENT RIVER-WATERS.

This comparison has been made by measuring the rate of oxidation of the constituents of sewage effluents or unpolluted river-muds in the presence of various river-waters. The sewage effluents were diluted with the river-waters, and the mixtures incubated at 18.3° C. usually for five days as in the dissolved oxygen absorption test. The amount of oxygen absorbed by the diluted sewage and also by the unpolluted river-water itself being thus determined by Winkler's process, the dissolved oxygen absorption due to the sewage or effluent could be ascertained by difference¹. The experiments with the muds were similarly carried out.

The geological source and nature of the river-bed has been investigated in the case of each river, and the conditions have been correlated with the results of the oxidation experiments. The results are set forth in the following table. Unless otherwise stated, the temperature of the incubation was 18.3°, dilution of effluent 1:5, and period of incubation five days.

¹ In the case of tank-effluents, the amounts used were so small and the dilution thus was so great that the actual absorption of dissolved oxygen by the river-water itself was not required, because the small amount of sewage did not affect the estimation of oxygen, and the absorption of oxygen by the tank-effluent could be determined by taking the difference between the oxygen concentrations of the river-water, and river-water + effluent at the end of the period of incubation. This was also the case in the experiments with muds, as before titration the supernatant water was syphoned off from the layer of mud into another stoppered bottle.

Comparison of rate of oxidation in river-waters.

Material used	River (1)			River (2)			
	Dissolved oxygen absorbed, parts per 100,000, by		Character of river-bed at point of sampling	Dissolved oxygen absorbed, parts per 100,000, by		Character of river-bed at point of sampling	
	Effluent when di- luted with this water	River- water alone		Effluent when di- luted with this water	River- water alone		
(a) 1. From Gravel and Upper Bagshot Sands.							
2. From same beds but after river has subsequently run over, first Bracklesham beds, Lower Bagshot and finally London Clay for a considerable distance.							
Land-effluent		1.35	0.19	Gravel and sand	1.70	0.02	Clay
"		0.55	0.19	"	1.85	0.02	"
"		0.00	0.19	"	0.95	0.02	"
"		2.61	0.00	"	2.19	0.11	"
"		2.68	0.00	"	2.05	0.11	"
"		0.94	0.00	"	0.67	0.11	"
"		1.04	0.00	"	0.68	0.11	"
"		1.05	0.09	"	0.95	0.19	"
"		0.60	0.09	"	0.55	0.19	"
"		1.05	0.18	"	1.60	0.23	"
"		1.20	0.08	"	1.10	0.53	"
"		0.65	0.00	"	1.15	0.22	"
"		0.80	0.04	"	0.65	0.13	"
"		1.60	0.04	"	0.45	0.13	"
Tank-effluent							
(dilution 1 in 50)	2 days	13.75	—	"	{ 36.25 } { 30.25 }	—	"
"	1 "	6.50	—	"	11.10	—	"
"	2 "	13.75	—	"	14.75	—	"
"	1 "	17.00	—	"	16.00	—	"
"	1 "	4.00	—	"	6.00	—	"
"	3 "	9.00	—	"	13.40	—	"
"	5 "	10.50	—	"	15.20	—	"
"	2 "	23.50	—	"	21.70	—	"
"	3 "	28.50	—	"	14.50	—	"
"	4 "	34.50	—	"	24.50	—	"
River-mud							
(dilution 1 in 100)		63.3	—	"	70.00	—	"
"		65.0	—	"	58.30	—	"
(b) 1. From Gravel and Upper Bagshot Sands.							
2. From same beds but mixed with river-water from Bracklesham beds.							
Land-effluent		0.35	0.13	Gravel and sand	1.05	0.24	Sand and clay
"		0.65	0.15	"	0.00	0.27	"
Tank-effluent							
(dilution 1 in 50)	2 days	22.25	—	"	10.75	—	Green (Glauc- conitic) clay
"	2 "	26.00	—	"	17.15	—	"
"	2 "	10.75	—	"	11.62	—	"
"	4 "	14.25	—	"	17.00	—	"
Glaucconitic mud from river in Bracklesham beds (dilution 1 in 100)		63.30	—	"	55.80	—	"
"		65.00	—	"	53.70	—	"
Land-effluent		0.65	0.09	"	0.00	0.11	Clay and sand
"		0.50	0.06	"	0.60	0.17	"
(c) 1. From Gravel and Upper Bagshot Sands.							
2. From Lower Bagshot, subsequently running over London Clay.							
Effluent		1.95	0.11	Gravel and sand	3.35	0.17	Clay
"		0.18	0.03	"	1.35	0.22	"

Comparison of rate of oxidation in river-waters.

(g) From Gravel, Sand, and Clay, and from the Chalk.

Material used		Dissolved oxygen absorbed, parts per 100,000, by			Dissolved oxygen absorbed, parts per 100,000, by		
		Effluent diluted with the river-water	River- water alone	Character of river-bed at point of sampling	Effluent diluted with the river-water	River- water alone	Character of river-bed at point of sampling
		River-water from Lower Bagshot Clay, subsequently running over London Clay.			River-water from Chalk subse- quently running over Wool- wich and Reading beds.		
Land-effluent		1.15	0.23	Clay	1.70	0.06	Calcareous gravel
„		0.90	0.23	„	0.80	0.06	„
		River-water from Lower Chalk.			River-water from Folkestone beds subsequently running over Sandgate beds.		
„		2.60	0.09	Marly loam and calcareous gravel	1.45	0.19	Sand
		River-water from Gravel and Up- per Bagshot Sands.			River-water from Chalk subse- quently running over Wool- wich and Reading beds.		
Tank-effluent	1 day	24.9	—	Gravel and sand	32.0	—	Calcareous gravel
(dilution 1 in 50)							
„	2 „	34.4	—	„	41.3	—	„
„	3 „	40.0	—	„	45.7	—	„
		River-water from Gravel and Up- per Bagshot Sands, subsequent- ly running over Bracklesham beds, Lower Bagshots and Lon- don Clay.			River-water from Chalk subse- quently running through Wool- wich and Reading beds.		
Tank-effluent							
(dilution 1 in 50)	1 day	11.40	—	Clay	6.00	—	Calcareous gravel
„	2 „	9.50	—	„	8.80	—	„
„	3 „	10.60	—	„	3.20	—	„
		River-water from Lower Bagshot subsequently running over Lon- don Clay.			River-water from Chalk subse- quently running over Woolwich and Reading beds (3 different rivers).		
Filter-effluent		1.35	0.22	Clay	1.15	0.05	Calcareous gravel
„		River-water from Gravel and Up- per Bagshot Sands.			0.75	0.02	„
„		0.18	0.03	Gravel and sand	1.08	0.11	„
		River-water from Gravel and Up- per Bagshot Sands.			River-water from Lower Chalk, Upper Greensand and Gault subsequently running over Folkestone beds.		
Tank-effluent							
(dilution 1 in 50)	1 day	9.50	—	Gravel and sand	10.25	—	Clay
„	2 „	11.25	—	„	19.87	—	„
„	3 „	16.75	—	„	22.70	—	„
		River-water from Folkestone beds subsequently running over Sandgate beds.			River-water from Lower Chalk, Upper Greensand and Gault subsequently running over Folkestone beds.		
Filter-effluent		11.40	0.16	Sand	9.85	0.11	Clay
		River-water from Sandgate beds, Lower Greensand.			River-water from Lower Chalk and Upper Greensand.		
Effluent		0.80	0.04	Sand	0.45	0.00	—

A survey of the tabulated results shows that very considerable differences exist in the rate of oxidation of the constituents of sewage effluents in different river-waters of different geological source. There is however a marked inconsistency in the results, so that it is not yet possible to draw any definite conclusions as to the influence of geological source on the oxidation processes in river-waters. This inconsistency may be expected, as the conditions, *i.e.* the chemical composition and bacterial population of sewage are subject to very great variation, and thus in each experiment new factors may be introduced.

The results so far obtained may be summarised thus:

Although in the case of nearly all the river-waters used for comparisons, close agreement in the rates of oxidation was sometimes observed, yet marked differences were frequently found.

Oxidation proceeded more slowly or more quickly in:

1. River-water from the Gravel and Upper Bagshot Sands than in river-water derived from the same source but subsequently running over successively the Bracklesham beds, Lower Bagshot Sands, and London Clay.

2. River-water from the Gravel and Upper Bagshot Sands than in river-water from the same beds, but mixed with river-water from the Bracklesham beds.

3. River-water from the Folkestone beds than in river-water from the Hythe beds.

4. River-water from the Folkestone beds than in river-water derived from the same beds but subsequently running over the Sandgate beds.

5. River-water from the Gravel and Upper Bagshot Sands than in river-water from the Lower Greensand strata.

6. River-water from Gravel, Sand, and Clay than in river-water from the Chalk.

7. Oxidation proceeded more slowly in river-water from the Gravel and Upper Bagshot Sands than in river-water from the Lower Bagshot Sands and London Clay.

A considerable amount of work has yet to be done in order to interpret these variable results. They indicate however the importance of always strictly using the particular river-water into which an effluent is discharged for dilution of the effluent in Winkler's dissolved oxygen absorption test. This rule should always be adhered to, unless it can be conclusively shown by a long series of experiments that any other river-water, which happens to be more convenient for use, gives results in close agreement.

No work seems to have been carried out previously on the deoxygenating action of the muds of unpolluted rivers. The experiments described in this paper show that such river muds can absorb from 12 to 70 parts per 100,000 of oxygen from river-waters in five days at 18.3° C.

Further observations are given below:

Character of mud	Supernatant water used	Dissolved oxygen absorbed. Parts per 100,000
From clean bed of stream formed by spring-water from Hythe		
beds thrown out by Atherfield Clay	Stream water	6.00
From stream in Folkestone beds	Distilled water	16.00
From river in Sandgate beds	"	40.00
From river in London Clay	River-water	44.00

It is seen that unpolluted river-muds absorb very considerable amounts of dissolved oxygen, and their potential deoxygenating effect is in every case much greater than that of a sewage effluent of fair quality (2 parts per 100,000). As a general rule however muds of unpolluted rivers will have actually less deoxygenating effect than sewage effluents, because the volume of the superficial deoxygenating layer of mud relative to that of the river is small.

7. COMPARISON OF THE DISSOLVED OXYGEN ABSORPTION OF SEWAGE EFFLUENTS WHEN DILUTED WITH VARIOUS RIVER- AND TAP-WATERS.

In a previous communication [Cooper, 1918] it was shown that the dissolved oxygen absorption figures of effluents were considerably lower when dilution was made with a hard tap-water from the Chalk than with distilled water or river-water taken from the river receiving the effluent. It was thought desirable to obtain more data bearing on this question and comparisons of the rates of oxidation in the presence of: 1. River-water above the outfalls. 2. Tap-water at laboratory. 3. Local tap-water, *i.e.* water-supply running into sewerage.

Some results on these lines have already been given in Section 6 of this paper, as the river-water from the Gravel and Upper Bagshot Sands happened to be derived from the same springs as yielded the laboratory tap-water and the results thus serve as comparisons of dissolved oxygen absorption in the presence of tap-water (Gravel and Upper Bagshots) and the river-waters.

It is seen that the oxygen absorption often varied considerably and quite different results were obtained according as tap- or river-water was used for dilution. Further comparisons are made in the table on p. 362.

It is seen that lower results were frequently obtained with the various hard calcareous waters, thus confirming the previous findings. This however was not invariably the case.

The fact that hard waters do not always inhibit the oxidation processes is further brought out by the following experiments with river-muds and effluents:

Supernatant water					Dissolved oxygen absorption mud or effluent. Parts per 100,000	
1.	Distilled water	16	
	Hard water from Chalk	12	
	"	20	
2.	Distilled water	40	
	" plus solid calcium carbonate	39	
	Hard water from Chalk	30	
					<i>a.</i>	<i>b.</i>
3.	Effluent diluted with distilled water	1.20	0.90
	" " " " plus Oolite	1.05	0.95
	" " " " plus Barytes	0.90	0.75

Dissolved oxygen absorption. Parts per 100,000 of effluent.

Diluted with river-water receiving effluent		Diluted with laboratory tap-water	Diluted with local tap- water, i.e. water-supply running into sewerage
1.	36.25	28.0	—
	30.25	23.0	—
	(London Clay)	(Hard water from Chalk be- neath Tertiary formations)	
2.	0.55		1.90
	(London Clay)		(Hard water from Chalk be- neath Tertiary formations)
	0.00	—	7.75
	(Gravel and Upper Bagshot Sands)		(Hard water from Chalk be- neath Tertiary formations)
	23.0	23.0	—
	(Gravel and Upper Bagshot Sands)	(Hard water from Chalk be- neath Tertiary formations)	
	0.00	0.65	—
	(Bracklesham beds)	(Gravel and Upper Bagshots)	
	0.90	—	1.15
	(Sandgate beds)		(Hythe beds)
	0.30	—	0.35
	(Gravel and Upper Bagshot and Bracklesham beds)		(Chalk beneath Tertiaries)
	20.7	19.5	—
	23.75	24.35	—
	(Gravel and Upper Bagshots)	(Gravel and Upper Bagshots)	
	3.35	1.95	2.80
	(London Clay)	(Gravel and Upper Bagshots)	(Chalk beneath Tertiary for- mations)
	1.30
			(Tap-water from another local supply, water from Chalk at surface)
	0.65	—	0.45
	(Bracklesham beds)		(Chalk beneath Tertiaries)
	1.90	—	1.05
	(London Clay)		(Chalk beneath Tertiaries and river gravel)
	1.35	0.18	0.90
	(London Clay)	(Gravel and Upper Bagshots)	(Chalk beneath Tertiaries and river gravel)
	1.05
			(Upper Chalk, another local supply)

8. COMPARISON OF DISSOLVED OXYGEN ABSORPTION OF SEWAGE EFFLUENTS DILUTED WITH RIVER-WATER AND DISTILLED WATER.

In the previous paper [Cooper, 1918] some evidence was offered showing that the dissolved oxygen absorption figures of sewage effluents were higher when distilled water was used for dilution than when river-water was em-

ployed. Since then many other experiments have been made and the results are recorded below:

Dissolved oxygen absorption. Parts per 100,000 of effluent.

1. Diluted with river-water receiving effluent		2. Diluted with dis- tilled water
0.0	(Gravel and Upper Bagshot Sands)	7.65
23.00	" " "	15.50
1.80	" " "	1.50
0.65	" " "	1.25
1.10	" " "	0.95
2.25	" " "	2.40
0.18	" " "	1.30
0.50	" " "	0.85
1.15	(London Clay)	1.25
1.35	"	1.30
2.05	"	2.00
2.35	"	2.75
0.80	(Sandgate beds—Lower Greensand)	0.45
1.25	" " "	1.25
1.95	(Hythe beds—Lower Greensand) ...	1.50
0.70	" " " ...	0.85
1.40	(Folkestone beds—Lower Greensand)	1.40
2.40	" " "	2.40
0.45	(Lower Chalk and Upper Greensand)	0.45
1.15	(Chalk and Tertiaries)	1.30
0.75	" "	1.30
1.08	" "	1.30

The results show that there is sometimes close agreement in the two sets of figures, and in this case for practical purposes it makes no difference whether distilled or river-water is used. In many cases however there is a considerable discrepancy and oxidation may go on either more slowly or more quickly in river-water than in distilled water. In the case of water from the Gravel and Upper Bagshot Sands the inhibitory effect was often very marked, and was greater in fact than shown in the earlier results [Cooper, 1918]. Oxidation also proceeded more slowly in river-waters from the Chalk than in distilled water.

River-water from the London Clay on the other hand as a rule had no perceptible inhibitory or accelerating effect on the oxidation processes. In one experiment however there was an appreciable inhibition. In the case of river-waters from the Lower Greensand (Sandgate, Folkestone, and Hythe beds) the results were variable. The earlier experiments [Cooper, 1918] pointed to a considerable inhibitory action, but in some of the further experiments described above oxidation proceeded either more rapidly in the river-waters or at the same rate approximately as in distilled water.

9. COMPARISON OF DISSOLVED OXYGEN ABSORPTION OF SEWAGE EFFLUENTS WHEN DILUTED WITH VARIOUS TAP-WATERS.

In the previous communication it was shown that the constituents of sewage effluents oxidised about 5 to 20 times more rapidly in distilled water than in a hard tap-water, and about 25 per cent. faster in distilled than in tap-water from the Gravel and Upper Bagshot Sands.

In the case of a highly ferruginous tap-water from the Folkestone beds there was an initial inhibition which however was no longer noticed when the iron was removed by oxidation and filtration. In the usual five days' dissolved oxygen test, on the other hand, oxidation proceeded at the same rate in the tap-water (filtered or unfiltered) as in distilled water.

Some further observations on the relative rate of oxidation in distilled water, hard tap-waters, and tap-water from the Gravel and Upper Bagshot Sands are given below:

Dissolved oxygen absorbed in five days at 18.3° C.

Material used	Dilution with		
	Distilled water	Hard water	Soft water from Gravel and Upper Bagshot
Primary filter-effluent	1.30	0.90	0.18
" "	1.30	1.05	0.18
" "	—	2.80	1.95
" "	—	1.30	1.95
Tank-liquor	—	7.75	0.00
"	—	23.00	23.00
Land-effluent	—	0.35	0.30

Additional figures affording comparisons of oxidations in distilled water and tap-water from Gravel and Upper Bagshot Sands are given in Section 8, the river-water from these geological formations being derived from the same springs as supplied the tap-water.

A survey of all the results shows that there is very great variation in the relative rate of oxidation of sewage constituents in distilled water and in the tap-waters (soft and hard). It is seen that both tap-waters frequently exercise a very considerable inhibitory effect on the rate of oxidation, and that the hard water may have either a greater or smaller inhibitory action than the soft water. In some cases however there is a fairly close agreement. The results point to the conclusion that the oxidation processes in the artificial methods of sewage purification must be considerably affected by the nature of the water-supply which mixes with the sewage, and also by that of surface-water gaining access to the drainage system. Owing to the great variations it is not possible to draw any helpful conclusions from the results of laboratory work in reference to this question, but it may be pointed out that the possibility of inhibitory action should always be borne in mind in framing new schemes for sewage purification, and arrangements should always be made to render it easily possible to extend the area of filtration, should difficulties be encountered.

10. THE OXYGEN ABSORBED FROM PERMANGANATE OR "TIDY TEST."

The test is used extensively in the chemical examination of water supplies. It is also employed to a certain extent in the standardisation of sewage effluents, although the Sewage Commission [*Roy. Comm.* 1913, p. 74] has reported that it gives a less correct measure of the polluting effect of an effluent than the dissolved oxygen test. Some observations made in the course of water analysis at first suggested that geological factors influenced the permanganate test just as they had been found to affect the Winkler's test. Thus, the loss in strength of the permanganate solution on incubation was occasionally found to be greater in the presence of distilled water than in presence of pure tap-waters (calcareous or soft water supplies).

10 cc. *N/80 potassium permanganate*. 10 cc. 25 % *sulphuric acid*.
100 cc. *water*. Four hours at 37° C.¹

Immediate titration.	Titration with thiosulphate after 4 hrs incubation with distilled water	Titration after 4 hrs incubation with pure tap-water
12.4 cc.	11.2 cc.	11.8 cc. (hard water)
15.0	13.2	13.8 "
15.4	15.0	15.3 (soft water)

It would seem that certain constituents of the tap-water inhibit either the natural decomposition of the permanganate or the oxidation of some impurity present in it [Cooper and Heward, 1919]. Experiments were therefore next carried out to ascertain if the rate of oxidation of the constituents of sewage by permanganate were affected by dilution with different tap-waters. For this purpose the Tidy Test was carried out in the usual way.

10 cc. *Tank Liquor (Sedimented Sewage)*. 10 cc. *N/80 permanganate*. 10 cc. 25 % *sulphuric acid*. 90 cc. *water*. Four hours at 37° C.

Oxygen absorbed by Tank Liquor from Permanganate. Parts per 100,000.

Dilution with distilled water	Dilution with hard calcareous water	Dilution with soft peaty water
3.10	3.00	3.50
2.00	2.10	—
7.25	7.40	—
8.00	—	8.20
3.70	—	2.33

The results as a whole show that the permanganate test unlike the dissolved oxygen test is not appreciably affected by the presence of a hard or soft water. It follows from this that the permanganate test gives a more correct measure of the actual amount of oxidisable matter present, and is thus of special value in water analysis. The dissolved oxygen test on the other hand determines the amount of oxidation that can go on under certain defined conditions, and thus measures, not the proportion of oxidisable matter present, but its actual deoxygenating or polluting effect on the diluting water. This test will thus be of special value in the standardisation of sewage effluents.

¹ 37° C. was used as standard temperature as it was more convenient and as there was practically no difference in the results obtained at 27° and 37°.

SUMMARY.

1. Fully aerated sewage effluents and polluted river-waters may contain considerably less dissolved oxygen than distilled water under the same experimental conditions. The low results appear to be due to various factors, and cannot be prevented by any method so far employed. As, however, the oxygen content is still the same after incubation for five days and subsequent re-aeration, there seems to be no appreciable error involved in Winkler's dissolved oxygen absorption test (Rideal-Stewart modification).

2. The oxidation of the constituents of sewage proceeds much more slowly at 10° C. than at the standard temperature of 18.3° C. usually employed.

At temperatures of 22° C. and 37° C. oxidation may proceed either at the same rate as at 18.3° C. or sometimes faster. The results show the necessity of adhering strictly to a definite temperature in the dissolved oxygen absorption test. The temperature of 18.3° C. was recommended by the Sewage Commission, this being the maximum temperature of river-waters in this country.

3. Attempts to shorten the five days' dissolved oxygen absorption test to two days have not been successful, as owing to an initial lag most variable ratios between the two and five days' tests are obtained. A bad effluent in fact may give very little indication of oxygen absorption in two days.

4. Dilution appears to have comparatively little influence on the rate of oxidation of the constituents of sewage.

5. Ammonia up to a concentration of 45 parts per 100,000 has also only a slight inhibitory effect on the rate of oxidation.

6. Oxidation of sewage constituents may proceed at very considerably different rates in river-waters of different geological source. The results are however very variable, and it is not yet possible to draw any definite conclusions as to the influence of geological factors upon the oxidation processes.

While in the case of nearly all river-waters employed, close agreement in the results is sometimes observed, oxidation may be slower or quicker:

(i) In river-water from the Gravel and Upper Bagshot Sands than in river-water from the London Clay, Bracklesham beds, and Lower Greensand formation.

(ii) In river-water from the Folkestone beds than in river-water from the Sandgate and Hythe beds.

(iii) In river-water from Gravel, Sand, and Clay formations than in river-water from the Chalk.

7. Mud from unpolluted rivers contains a considerable amount of oxidisable matter and may have a deoxygenating effect upon the water from 3 to 35 times as great as that of a good standard sewage effluent. The actual volume of the superficial deoxygenating mud relative to that of the supernatant water being small, however, muds from clean rivers will in general have less polluting effect than an effluent.

8. Considerably lower or higher results in the dissolved oxygen absorption test may be obtained when tap-water from the Gravel and Upper Bagshot

Sands is used for dilution than when the river-water into which the effluent actually drains is employed. Sometimes however there is close agreement between the two sets of figures.

9. Lower results are often obtained when hard calcareous tap-waters are used for dilution instead of the river-waters. Occasionally however the results are higher, while sometimes there is no appreciable difference noticed.

10. There may sometimes be close agreement in the dissolved oxygen absorption figures of an effluent diluted respectively with river-water and distilled water.

In many cases however, *e.g.* river-water from the Gravel and Upper Bagshot Sands, Lower Greensand, and Chalk, there is a considerable difference, and oxidation may go on more slowly or more rapidly in the river-water than in distilled water; the river-water thus having an inhibitory or accelerating action as the case may be.

In the case of river-water from the Gravel and Upper Bagshot Sands, and the Chalk, the *inhibitory* action upon the processes of oxidation may be especially marked.

11. The main conclusion to be drawn from the results summarised in 6-10 is that in the standardisation of sewage effluents the river-water into which the effluent is actually discharged must be used for dilution in Winkler's dissolved oxygen absorption test, unless it can be conclusively shown by several experiments that reliable results can be obtained by employing a more convenient diluting medium, *e.g.* another river-water, tap-water, or distilled water.

12. It is very likely that the oxidation processes in artificial methods of sewage purification may be affected by the nature of the water-supply, and by surface water entering the drainage system. Both hard and soft tap-waters may exert a very considerable inhibitory action in laboratory experiments. It is recommended therefore that allowance for possible inhibitory factors be always made in preparing new schemes of sewage purification, so that additional filtering area can be subsequently added, if necessary, with the minimum of trouble and expense.

13. The oxidation by permanganate or Tidy Test, unlike the dissolved oxygen absorption test, does not appear to be affected by the presence of waters of different geological source.

A consideration of the facts leads to the conclusion that the permanganate test is more suitable for the chemical examination of water supplies, while the dissolved oxygen test is more suitable for the standardisation of sewage effluents.

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XXXV. ON THE DIGESTIBILITY OF COCOA BUTTER. PART I.

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THERE is a considerable literature dealing with the value of cocoa as a food, and a number of observers describe experiments, carried out both on man and animals, to test the physiological utilisation of the fats and proteins contained in it. In spite of the fact that the fat is of high melting point, and is stated to be resistant to enzyme action *in vitro*, it appears, when cocoa itself is eaten, to be well absorbed and utilised. The proteins are on the whole less available, but in the majority of the experiments recorded they show a fairly satisfactory utilisation.

H. Cohn [1895] in experiments carried out upon himself found that over 95 % of the fat was absorbed, but only about half the protein. R. O. Newmann [1906] in prolonged experiments got favourable results, and found that, when moderate quantities were used, the utilisation of the fat was about 90 %. Similar results were obtained by V. Gerlach [1907]. L. Pincussohn [1907], who added 35 g. of cocoa per day to the diet of a number of subjects, found that the fat absorption was very good, in some cases as high as 98 %. The protein absorption was, however, variable, ranging from 60 to 90 %, but was best in preparations containing a low percentage of fat. N. Zuntz [1890] in the case of chocolate found that 95 % of the fat was utilised. None of these observers noticed any ill-effects upon health as the result of taking for considerable periods quantities in excess of what would be consumed in ordinary life.

Until recently there were comparatively few experiments recorded on the feeding of the actual fat contained in cocoa-beans, namely cocoa butter. Zuntz in the above-mentioned paper, in a very extensive series of observations, found that dogs fed with cocoa butter absorbed about 90 %, a utilisation very nearly as good as in the case of bacon fat. Langworthy and Holmes [1917] published a paper on the digestibility of some vegetable fats, and amongst these fats cocoa butter was included. These experiments were conducted by similar methods to those that were employed in a previous investigation [1913] on animal fats. A basal ration, supplying a minimum of fat, composed of wheat biscuits, oranges, sugar and tea, or coffee if desired, was supplemented by a blancmange or corn-starch pudding, in which was incorporated the vegetable fat under consideration. This diet was fed to normal young men, in

good health and moderately active, for periods of *three* days. Weighings were made of nett amounts of food eaten and faeces excreted, and samples of both food and faeces were analysed to determine the percentages of protein, fat and carbohydrate which were actually digested.

The digestibilities of olive oil, cotton seed oil, pea nut oil, coconut oil, sesame oil and cocoa butter were in this way compared. In 11 experiments using different amounts of cocoa butter, the average percentage utilisation was 89.6. The figures however were rather variable, running from 79.1 % to 95.5 %.

The fat-carrying blancmange was *not relished in the cocoa butter experiments* as it was in the case of the other fats, for the amount eaten was only enough to supply 51 g. of cocoa fat per man per day during the first eight experiments. This quantity did not cause any decided digestive disturbance so far as was noted. The subjects reported a "loss of appetite," but in all other respects considered that their physical condition had been normal. In later tests the subjects were urged to eat more of the blancmange containing the cocoa butter. As a result of eating the larger quantities, an average of 109 g. per day, undesirable physiological derangements were experienced. The effects were so pronounced that one subject discontinued the diet at the end of the seventh meal. "Loss of appetite," "headache," "loss of ambition," "nausea" and "sleeplessness" were conditions reported, which, the authors consider, indicate that cocoa butter in quantity had an effect not noted in the case of the other vegetable fats studied. Though the exact limit of tolerance was not determined, to judge by the experiments made in the laboratory, the maximum amount of cocoa butter that can be consumed daily without decidedly unpleasant effects lies between 51 and 109 g.

The digestibility of the carbohydrate and protein did not seem to be influenced by the digestibility of the fat. In the experiments in which large quantities of the fat were fed, the faeces contained comparatively large quantities of fat—in one experiment as much as 37 % of the dry weight. In this case they state that an odour suggesting that of cocoa butter could be clearly detected.

This latter observation is however misleading, as a smell of cocoa is in our experience a normal constituent of the smell of all faeces. Dry faeces, after extraction with ether, retain this component of the smell in a marked degree, and it has nothing to do with the nature of the diet.

Messrs Langworthy and Holmes give the following figures for the coefficients of digestibility of the oils examined. In arriving at these numbers an allowance for metabolic products is introduced.

Olive oil	97.8
Cotton seed oil	97.8
Pea nut oil	98.3
Coconut oil	97.9
Sesame oil	98.0
Cocoa butter	94.9

With regard to cocoa butter they conclude that in view of the unsatisfactory utilisation of this fat and the accompanying physiological disturbances, the advisability of the continued daily use of cocoa butter in large amounts would appear questionable.

NATURE OF THE PRESENT INVESTIGATION.

The object of the experiments detailed below was to arrive at an opinion as to whether cocoa-butter could be utilised with economy as part of the fat supply of the population without detrimental results to health.

In studying the digestibility of a single food, experience has shown that it is advisable to incorporate it in a simple mixed diet, since the ordinary individual is so accustomed to a mixed diet that, no matter how palatable a simple food may be at first, it soon becomes distasteful. It is also essential that the diet in question should be palatable, and we think it desirable that no constituent of the diet should be very largely in excess of the physiological balance. This was obviously not the case in the American experiments in which large quantities of cocoa butter were fed. The diets made use of by the sub-committee of the Royal Society in their experiments on the digestibility of breads made from different kinds of flour [1918] seemed very suitable for the purpose, and in order to save time it was decided to utilise the material accumulated in these experiments as controls.

The Diets consumed.

The details of the control diets have been published in the report by the Food (War) Committee of the Royal Society on the Digestibility of Breads [1918]. Approximately the daily diet consisted of

Bread	800-1000 g.
Minced meat	50 "
Butter	50 "
Jam	100 "
Milk	600 cc.
Cheese	50 g.
Sugar	30 "
Tea	<i>ad lib.</i>

In the tables in this paper the control diets, the fat content of which has not hitherto been published, are referred to as A, B, and C. In A the bread was made from 80 % milled flour, in B from 90 % milled flour and in C from 80 % milled flour mixed with 20 % maize. Almost the same diet was used in the cocoa butter experiments, substituting 50 g. of cocoa butter for the 50 g. of butter. The bread used in these latter experiments was the ordinary bread of the shops, and the daily ration was about 850 g. It was not possible to use the special breads of the control diets, but the results of the bread experiments warrant the assumption that the use of ordinary shop bread would have little

or no influence on the results. However, to obviate this as far as possible each subject was instructed to purchase his own bread.

Probably the results are more strictly comparable with diet C, the bread of which was made from mixed cereals, since the bread supplied to the public at the time of the cocoa butter experiments was of this nature. The cocoa butter used was the ordinary fat sold under that name.

It was rather hard and brittle, pale yellow in colour and possessed when fresh a distinct flavour of cocoa, which however rapidly diminished in intensity on keeping even in a closed vessel.

It consisted almost entirely of pure fat, and the constants given below are well within the limits of variation given in the literature.

Moisture	0.17 %	Melting point	33.5 ° C.
Ash	0.059 „	Acid value	1.98
Unsap. matter	0.907 „	Sap. value	194
Sterols	0.171 „	Reichert-Meißl value	0.84
		Iodine value	37.3

Subjects used in the experiments.

An account of the eight subjects, four in Cambridge and four in London who had the control diets is given in the above-mentioned report. Out of the four subjects, previously experimented on in London, two only, viz. E. and B., were still available for the cocoa butter experiment, the others having been called to the colours. We were only able to get one additional subject—P.

Individual	Age	Wt in kilos	Occupation and state of health
E.	36	55	Laboratory assistant. Delicate health
B.	34	65	Laboratory attendant. Athlete and active worker
P.	18	64	Laboratory attendant. Active and in good health

E. and B. were therefore the only subjects who had both the control and the cocoa butter diets.

EXPERIMENTAL METHODS.

The general procedure was exactly the same as that detailed in the Royal Society Report on the Digestibility of Breads, the diet being consumed for a period of ten days, the six significant days being the 4–9th inclusive. Accurate accounts of the weights of food eaten were kept and analyses of each food were made. On the fourth, sixth and ninth days a determination of the nitrogen in the urine was also made.

As before, the collection of faeces began one day later and continued one day longer than the days of the diet.

Methods of analysis.

The coefficients of digestibility given in Langworthy and Holmes' paper referred to above were obtained in the customary way by analysing the food eaten and the faeces excreted during an experimental period. For this purpose

the total ether extract of the faeces was taken to represent the actual quantity of undigested fat—the unavailable residual of the fat eaten during the experimental period. That this assumption is far from true is apparent from a study of the composition of the faeces. It is well known that the faeces contain not only undigested food, and undigested residues of food, but also other waste materials, usually grouped under the somewhat ambiguous term metabolic products, some of which are soluble in ether. The ether extract, usually considered as fat, consists of true fat, fatty acids, and unsaponifiable matter. The unsaponifiable matter consists mainly of a variety of alcohols of the sterol group with a small proportion of fatty alcohols of lower molecular weight such as cetyl alcohol, etc. This unsaponifiable matter is partly metabolic in origin and is partly contained in the foodstuff. The ratio of true fat to fatty acids is variable, but as a rule the quantity of true fat is small and is often negligible. Faeces also contain salts of fatty acids, or soaps which are insoluble in ether.

It was therefore considered necessary to submit both the food and faeces to a more detailed analysis than is usually the case.

The faeces were analysed in the following manner. A weighed portion of the dried faeces was subjected to a prolonged extraction (not less than a week) in a Soxhlet apparatus with ether, and the ethereal solution was made up to known volumes. Aliquot portions were then respectively titrated with standard alcoholic soda, and evaporated to dryness and weighed. In the latter estimation care must be taken not to overheat. The rest of the ethereal solution was then mixed with an alcoholic solution of sodium in considerable excess and allowed to stand 48 hours. The precipitated soaps were then filtered on the pump and thoroughly washed with ether. The ethereal filtrate and washings were then shaken with alkaline water and finally with distilled water until quite free from soap. The ethereal solution of unsaponifiable matter thus obtained was made to known volume and an aliquot portion evaporated and weighed. Estimations of the sterols, precipitable by digitonin, in the unsaponifiable residue were also made, but these values will form the subject of a separate communication. In order to estimate the fatty acids in the form of soaps, the faecal residue after extraction with ether was moistened with an alcoholic solution of hydrochloric acid, dried and again extracted with ether. In this case the acids were not estimated by evaporating the ethereal solution, as it was thought that the more soluble fatty acids being stronger than those of high molecular weight would mainly be found in these soaps. The ethereal extract was therefore exactly neutralised by a standard alcoholic soda, evaporated to dryness and weighed.

Fat in the foods was determined by extraction with ether in the usual way, and the unsaponifiable matter and sterols estimated as described. In the case of bread and meat the ether extraction was preceded by extraction with boiling alcohol, as it is well known that in such substances the extraction of fat by ether alone is imperfect, however long the extraction may be prolonged. The results are given in the following tables.

The percentage availability of the nitrogen of Diet D per day and also the nitrogen balance per day.

		E.	B.	P.	
Nitrogen	Intake in food ...	16.51	20.35	19.87	
	Excreted in urine ...	13.91	13.47	12.99	
	„ in faeces ...	2.12	2.80	2.78	
	Total ...	16.03	16.27	15.77	
	Nitrogen absorbed ...	14.39	17.55	17.09	
	Balance ...	+ 0.48	+ 4.08	+ 4.10	
	Percentage utilisation	87.14	86.2	86.0	Average 86.4

		London subjects				Cambridge subjects			
		E.	C.	B.	A. C.	H. W. H.	F. J.	W. J.	A. L.
Total ether extract	Intake in food	98.93	97.28	99.41	92.79	155.28	92.53	122.08	139.93
	Excreted in faeces	3.52	3.66	4.33	3.11	4.94	1.63	2.47	2.32
	Difference	95.41	93.62	95.08	89.68	150.34	90.90	119.61	137.61
	% utilisation	96.44	96.24	95.65	96.65	96.81	98.23	97.98	98.34
		Average = 96.24				Average = 97.84			
		Average = 97.04							
True fat in food Fat excreted in faeces in form of true fat, fatty acids and fatty acids in form of soap	Intake in food	96.62	94.96	97.01	90.73	150.94	89.75	119.58	137.22
	Excreted in faeces	5.08	2.76	3.37	2.34	7.14	2.29	3.88	2.48
	Difference	91.54	92.20	93.64	88.39	143.80	87.46	115.70	134.74
	% utilisation	94.74	97.07	96.53	97.42	95.27	97.45	96.76	98.19
		Average = 96.44				Average = 96.92			
		Average = 96.68							

		London subjects				Cambridge subjects			
		E.	C.	B.	A. C.	H. W. H.	F. J.	W. J.	A. L.
Total ether extract	Intake in food	101.83	94.50	107.40	105.40	155.59	113.69	107.71	144.70
	Excreted in faeces	2.77	—	1.74	2.13	3.38	3.71	2.63	4.97
	Difference	99.06	—	105.66	103.27	152.21	109.98	105.08	139.73
	% utilisation	97.28	—	98.38	97.98	97.83	96.74	97.56	96.57
		Average = 97.88				Average = 97.18			
		Average = 97.48							
True fat in food Fat excreted in faeces as fat, fatty acids and fatty acids in form of soap	Intake in food	99.16	92.23	104.47	102.70	151.06	110.31	105.05	140.55
	Excreted in faeces	4.41	—	2.52	3.78	5.38	5.77	3.53	6.69
	Difference	94.75	—	101.95	98.92	145.68	104.54	101.52	133.86
	% utilisation	95.55	—	97.59	96.32	96.44	94.77	96.64	95.24
		Average = 96.49				Average = 95.77			
		Average = 96.08							

Table IV.

Diet C.

		London subjects				Cambridge subjects			
		E.	C.	B.	A. C.	H. W. H.	F. J.	W. J.	A. L.
Total ether extract	Intake in food	98.23	97.75	99.01	91.03	167.30	109.50	109.70	163.50
	Excreted in faeces	3.73	4.17	3.49	3.57	4.06	2.50	2.41	4.12
	Difference	94.50	93.58	95.52	87.46	163.24	107.00	107.29	159.38
	% utilisation	96.20	95.73	96.48	96.08	97.57	97.72	97.79	97.48
		Average = 96.12				Average = 97.64			
		Average = 96.88							
True fat in food	Intake in food	96.85	95.44	97.46	89.76	163.30	106.10	107.24	159.53
Fat excreted in faeces in form of true fat, fatty acids and fatty acids in form of soap	Excreted in faeces	4.36	6.54	3.72	3.59	4.06	2.50	4.12	2.41
	Difference	92.49	88.90	93.74	86.17	159.24	103.60	103.12	157.12
	% utilisation	95.50	93.15	96.18	96.00	97.51	97.64	96.16	98.49
		Average = 95.20				Average = 97.45			
		Average = 96.32							

Table V.

Diet D.

				London subjects			
				E.	B.	P.	
Total ether extract	Intake in food	...	109.00	105.73	105.53
			Excreted in faeces	...	5.41	6.66	6.66
			Difference	...	103.59	99.07	98.87
			Percentage utilisation		95.04	93.70	93.68
				Average = 94.14			
True fat in food	Intake in food	...	107.77	104.40	104.25
Fat excreted in faeces in form of true fat, fatty acids and fatty acids in form of soap	Excreted in faeces	...	6.27	7.56	7.62
			Difference	...	101.50	96.84	96.63
			Percentage utilisation		94.18	92.76	92.69
				Average = 93.21			

Table VI.

Comparative table showing average percentage fat utilisation in different diets.

						Diets			
						A	B	C	D
Total ether extract	97.04	97.48	96.88	94.14
True fat in food, and excreted in faeces as neutral fat, fatty acids and fatty acids in form of soap	96.68	96.08	96.32	93.21

HEALTH OF THE SUBJECTS.

All the subjects found the cocoa butter palatable, and were quite content with the diet. They suffered no appreciable change in weight during the experimental period. Their excreta were normal in appearance, and no apparent digestive trouble was noted. Their health was normal throughout.

DISCUSSION OF RESULTS.

Assuming that the bread sold to the public at the date of the experiments (1918) is comparable with that used in the diets A, B and C, it will be seen from Table I that in the case of the cocoa butter diet D there is a small but definite decrease in the utilisation of nitrogen, a result in agreement with observations recorded by various observers in experiments with cocoa.

The average percentage utilisation of fat in the cocoa butter diet D, whether judged by the total ether extract or by the true fat, is lower than the average values in the butter diets A, B and C, but the difference is not very marked, and the figures come within the limits of variation of A and C. The utilisation from this point of view must be regarded as satisfactory.

It will be noticed that the figure we obtained for the percentage utilisation of ether extract in diet D (94.14) is considerably higher than the figure 89.6 given by the American observers, though it comes within their limits of variation 79.1 to 95.5. It must be remembered however that diet D contained in addition to the cocoa butter a considerable amount of ordinary fat present in the meat, milk and cheese. That this slightly lower average utilisation is real and not merely the result of the smaller number of subjects eating diet D, and indicates that the cocoa butter is less digestible than ordinary butter, is shown by a consideration of the composition of the fatty matter of the faeces.

If we knew the average molecular weight of the acids excreted in the faeces on the various diets, it would be possible from the data we obtained to calculate for each individual the ratio of the fatty acids to unsaponified neutral fat in the faeces, since estimations were made on the ether extracts of (a) total solids by evaporation, (b) unsaponifiable matter, (c) free acids by titration with standard alkali: the difference between (a) and (b + c) would give the neutral fat. We do not know the average molecular weights of the acids in question, and furthermore the values for total solids may be slightly lower than the true values owing to loss of volatile acid on evaporation of the ether. We could not therefore get an accurate quantitative relation between the fatty acids and neutral fats in the faeces, but by assuming that the fatty acids were all stearic it can be shown that there is a marked difference between the composition of the faeces A, B and C on the one hand, and D on the other. This will be clear from the following figures for the two subjects who had all four diets.

Table VII.

Weights per cent. of dry faeces.

Diet	Individual	Total ether extract	Unsaponifiable matter	Real fatty matter	Approximate amount of fatty acid reckoned as stearic acid	Neutral fat by difference
A	E.	13.13	3.95	9.18	11.71	Probably nil
	B.	14.36	3.58	10.78	10.31	0.47
B	E.	6.99	2.40	4.59	8.37	Probably nil
	B.	5.50	1.72	3.78	6.28	"
C	E.	12.07	3.30	8.77	" 15.9 "	"
	B.	13.14	3.25	9.89	10.1	"
D	E.	15.18	1.96	13.22	1.41	11.81
	B.	15.48	1.80	13.68	4.05	9.63
	P.	14.07	0.90	13.17	4.54	8.63

It will be seen that whereas in the diets A, B and C the fatty matter of the ether extracts was mainly acids with little or perhaps no neutral fat, in the case of diet D the fat of the ether extract was mainly neutral fat. This clearly indicates that some small portion of the cocoa butter passed the intestinal tract unchanged.

More light would however be thrown on this question by a knowledge of the actual components of the fats used in the various diets, especially cocoa butter which differs markedly from animal fats and the more liquid vegetable fats, and of the fatty matter in the faeces produced. Experiments are being made on the separation of the acids from these various substances by fractional distillation of their methyl esters, with a view to the determination of the molecular weights, iodine values, etc. of the different fractions. These results are nearing completion and we hope shortly to be able to publish them.

QUALITATIVE EXPERIMENTS.

In order to get some idea of the tolerance for cocoa butter, and to find whether physiological disturbances similar to these noted by the American observers were produced when it was fed in excessive quantity with ordinary diet, four workers in the laboratory volunteered to try and eat three to four ounces per day for four days or so.

The four subjects were F. W. F. a chemist, W. L. S. a physiologist, W. G. R. a cadet of the O.T.C., and G. T. W. a cadet of the O.T.C.

They report as follows:

G. T. W. When I first started eating cocoa butter I did not enjoy it at all—that was eating about four ounces per day—but after the first two days I quite liked it, and found it most palatable when eaten with bread and jam. It has had no effect on me at all. The amount eaten was 1 lb. in five days.

W. G. R. Quantity eaten per day three to four ounces. At first the cocoa butter was unpleasant, giving rise to a bilious feeling, but later, when taken slightly warm with salt, this was not noticeable.

So long as it was eaten with some strong flavouring it made a fairly good substitute for butter, but was not pleasant when eaten alone with bread.

In spite of the fact that the amount taken was far in excess of my usual butter allowance, no ill effects were experienced.

W. L. S. I could not stomach much of your cocoa butter. I managed one ounce the first day, and was troubled by eructations. It also proved distinctly laxative. On the second day I could only take $\frac{1}{2}$ ounce and since then have taken none. It did not appear to be a very good sample¹.

F. W. F. I deliberately ate even larger quantities of cocoa butter than those given by the American observers, but without experiencing any of the unpleasant physiological effects they mention. The weights taken for the five days were as follows: 56, 237, 201, 102 and 135 g. These quantities were very far in excess of my ordinary fat ration, and were naturally unpalatably large. The faeces showed the high value, for the ether extract, of 24.3 g. % of dry weight.

GENERAL CONCLUSIONS.

1. Cocoa butter is rather less digestible than butter, but the experiments recorded show satisfactory utilisation.

2. Beyond the slight laxative action observed in the case of some of the subjects who consumed large quantities of the cocoa butter, no undesirable physiological effects were noticed.

3. Our experiments show no indication whatever that cocoa butter is a "slow poison," and there can be no doubt that this substance could be used to supplement the fat need of the population with safety. This conclusion is in agreement with general experience, since large quantities of this fat must have been consumed with impunity in the various chocolate preparations on the market—which may contain as much as 20 % of fat.

This work was undertaken at the request of the Food (War) Committee of the Royal Society, by whom the expenses were defrayed.

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¹ The same cocoa butter was supplied to each volunteer, and its constants were normal.

XXXVI. A NEW METHOD FOR PREPARING ESTERS OF AMINO ACIDS. COMPOSITION OF CASEINOGEN.

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IN a preliminary paper [Foreman, 1912] reasons were given for attempting to improve the methods commonly in use for separating the products of protein hydrolysis, and a new method for preparing the esters of the amino acids was briefly described.

The method has been applied to the products of two further hydrolyses of caseinogen with the following objects in view;

A. Hydrolysis by sulphuric acid. To determine the distribution of the nitrogen in the various fractions, and to test the completeness of the esterification.

B. Hydrolysis by hydrochloric acid. To determine the effect of the process upon the yields of the monamino acids, and to endeavour to throw some light upon the nature of the unidentified substances making up the deficit in protein analysis.

Before describing the experiments, it will be advisable to set forth briefly the steps involved. Full details of the working will be found in the account of Hydrolysis *B*.

(*a*) The amino acids are converted into their dry lead salts.

(*b*) The lead salts are suspended in absolute alcohol, and the esterification brought about by saturating with dry hydrochloric acid gas.

(*c*) The free hydrochloric acid is removed, partly by reducing the liquid to half its bulk at 40° and 15 mm., and the remainder by the addition of absolute alcohol saturated with dry ammonia gas.

(*d*) Alcohol is removed by evaporation at 40° and 15 mm., and the ester hydrochlorides taken up in dry chloroform.

(*e*) The esters are liberated from their hydrochlorides, and any remaining traces of esterification water removed by shaking the chloroform solution with anhydrous baryta.

(*f*) The chloroform is evaporated at 40° and 15 mm., and the esters taken up in anhydrous ether.

Hydrolysis A.

Nitrogen distribution. 320 g. "Hammersten casein," containing 45.056 g. N equal to 288.4 g. pure caseinogen calculated on the basis of 15.62 % N, was hydrolysed by boiling for 48 hours with three parts of sulphuric acid and six parts of water. The sulphuric acid was removed by means of baryta before making the lead salts.

The following table shows the percentages of the total nitrogen found in the fractions:

In the crude tyrosine removed	1.98
In the barium sulphate precipitate after very thorough washing	7.13
In the aqueous solution of amino acids	88.38
Loss	2.51
							100.00

From the aqueous solution of amino acids:

Ammonia lost when making the lead salts (calculated from the percentage for caseinogen given by Osborne and Guest [1911])							8.48
In the undissolved lead oxide and hydroxide							1.36
In the dry lead salts							77.08
Loss	1.46
							88.38

From the lead salts:

In the washed lead chloride removed after the esterification	1.63
In the filtrate containing the ester hydrochlorides	75.02
Loss	0.43
					77.08

The baryta residue obtained when the esters were liberated from their hydrochlorides contained 13.42 %, and a small quantity of esters soluble in chloroform but insoluble in ether 2.06 % of the total N. When removing the ether by distillation *in vacuo* from about half the ester solution, the flask was slightly fractured by accident and water gained access to the solution. The esters were recovered as far as possible by ether extraction, but small losses undoubtedly occurred. The nitrogen determinations were therefore discontinued at this stage. After removing the ether the esters weighed 244.7 g.

The ester process was not applied a second time to the material taken from the chloroform solution of the ester hydrochlorides by the anhydrous baryta. The quantity of esters obtained therefore represents the yield of a single preparation.

The esters were fractionally distilled in the usual way, and the fractions partially worked up. The quantities of amino acids separated, so far as these operations were carried, are given in the following table:

Amino acids	Total amounts obtained from 288.4 g. caseinogen. Obtained by one esterification
Glycine	0.5 g. triglycylglycine ester
Alanine, leucine and valine	52.09 g.
Proline	20.12 g.
Phenylalanine	8.96 g. of the hydrochloride
Aspartic acid	4.2 g.

The proline was estimated by van Slyke's method [1911].

The 8.96 g. phenylalanine hydrochloride represents the first crop of the crystals. The mother liquor would have yielded a further quantity. Phenylalanine ester was undoubtedly extracted to some extent by the water which gained admission to the ethereal solution of the esters by accident (p. 379).

The aspartic acid was separated as barium aspartate from the highest boiling ester fraction.

The 0.5 g. triglycylglycine ester separated from Fraction I while it was standing in the ester condition in a corked bottle for about three weeks. The substance was easily soluble in water, alkaline to litmus, and gave a pronounced biuret reaction. When its melting point was taken, it commenced to turn brown at 200°, sintered on further raising the temperature, blackened and finally decomposed at 267°. Fraction I always contains ether. The conditions were therefore identical with those under which Curtius [1904] prepared his biuret base, triglycylglycine ester. The composition, properties and reactions of the substance were also in agreement. Found 20.6 % N; calculated for triglycylglycine ester 20.44 % N.

The efficiency of the esterification method.

The dry lead salts weighed 558 g. and contained 50.27 % Pb. The lead chloride removed by filtration after esterifying, dried at 100°, weighed 335.7 g. The filtrate was found to yield a precipitate on adding absolute alcohol, which settled to the bottom in the form of a brown syrup. Extraction with a little cold water gave a brown solution containing 2.727 g. N leaving 36.2 g. lead chloride. The brown solution invites further investigation. The total weight of dry lead chloride recovered was therefore 371.9 g., which corresponds to 277 g. lead. The lead salts contained 280.5 g. lead. The lead salts were therefore practically completely transformed in the esterification.

Hydrolysis B.

YIELDS OF MONAMINO ACIDS. INVESTIGATION OF THE RESIDUES.

In the previous sulphuric acid hydrolysis, no less than 7 % of the total N was retained by the washed barium sulphate precipitate formed when the acid was removed. In order to avoid the effect of this loss upon the yields of amino acids, this hydrolysis was brought about by means of hydrochloric acid. Glutamic acid was removed in the form of its hydrochloride, as far as possible, before the esterification.

320 g. of the same sample of "Hammersten casein" as used in Hydrolysis A (= 288.4 g. pure substance) was hydrolysed in the usual way by boiling with hydrochloric acid for 48 hours.

Removal of glutamic acid as the hydrochloride. The liquid arising from the hydrolysis was evaporated on the water-bath to a volume of about 700 cc., saturated with hydrochloric acid gas, and left in the ice chest. The crystals

were filtered off on an asbestos pad, and washed several times with aqueous hydrochloric acid saturated with the gas at 0°. The crude hydrochlorides thus obtained weighed 88.1 g. after drying to constant weight. This material was taken up with water, the solution decolorised by warming with animal charcoal, reduced to about 300 cc., and saturated with hydrochloric acid gas. The perfectly white crystals were filtered off on asbestos, and washed many times with ice cold saturated aqueous hydrochloric acid. The free hydrochloric acid was removed in a desiccator over strong potash. 57.1 g. resulted after drying to constant weight at 100°. The filtrate yielded a further 0.16 g. of the hydrochloride when reduced in bulk and again saturated. The total weight was therefore 57.26 g., which corresponds to 45.87 g. glutamic acid. Portions weighed out from the whole specimen gave the following results on analysis:

	Found	Calculated
	%	%
C	32.61	32.70
H	5.80	5.45
N	7.64	7.62
Amino N (van Slyke)	7.74	7.62

It will be noted that the recrystallised material only accounted for about two-thirds of the crude dry hydrochlorides.

Preparation of the lead salts. The filtrate from the recrystallised glutamic acid hydrochloride was added to the main hydrolytic liquid, and hydrochloric acid removed as far as possible by evaporation on the water-bath. A current of steam was then passed through the heated fluid for some time to remove further hydrochloric acid. The volume was made up to 2 litres with water, a current of steam passed through, and 100 g. precipitated lead hydroxide added. The steaming was continued for a few minutes, and the hot solution filtered by suction. The insoluble material was dark in colour, and contained the greater part of the humin matter. 400 to 500 g. ordinary litharge was added to the filtrate in portions during a further 45 minutes' steaming. The excess of litharge was filtered off rapidly by suction, and washed with hot water.

The preliminary treatment with lead hydroxide was introduced because the compound formed with the humin matter remains in suspension, and can be easily filtered off. When litharge was used from the commencement, however, the humin compounds tended to collect at the bottom in the form of a troublesome cake. The use of litharge after lead hydroxide appears to be necessary for the complete transformation of the amino acids into lead salts, and a good excess should be added as the bigger particles are not readily dissolved.

The solution containing the lead salts was evaporated on the water-bath to a volume of about 500 cc. and cooled. The semi-solid mass of salts which resulted was placed on the water-bath and stirred until it became too viscous. After standing in the steam-oven all night and then being cooled the material can be easily reduced to a powder. The grinding however is quite unnecessary,

as the dry cakes will esterify readily when treated in the manner to be described. The dry lead salts weighed 475 g. The undissolved lead hydroxide and litharge contained 3.1 % of the total N of the 320 g. caseinogen.

Esterification. The lead salts were suspended in 1500 cc. absolute alcohol, and saturated with dry hydrochloric acid gas. The liquid was then cooled by means of a freezing mixture of ice and salt, and again brought to saturation. The lead chloride was filtered off and washed with absolute alcohol.

Removal of the free hydrochloric acid. The greater part of the free hydrochloric acid was removed by reducing the volume to one-half at 40° and 15 mm. The fluid was transferred to an enamelled jug, and the flask washed out with absolute alcohol. The jug was surrounded by a freezing mixture of ice and salt, and the remaining free hydrochloric acid removed by means of a saturated solution of dry ammonia gas in absolute alcohol, which was added slowly, with continual stirring, until the liquid remained only faintly acid. The further addition of alcoholic ammonia would liberate esters from their hydrochlorides, and the fluid would darken in colour. It was considered advisable, however, to stop at the faintly acid stage in order to avoid loss which might result from decomposition of the free esters in this as well as subsequent operations. By the means described the free acid was eliminated without involving the formation of water. The ammonium chloride was filtered off by suction, and washed thoroughly with absolute alcohol.

Removal of the alcohol. The alcohol was removed as completely as possible by evaporation at 40° and 15 mm. The specific gravity of the distillate was found to be higher than that of absolute alcohol. Assuming this increase to be due to the presence of water, the distillate contained a quantity which corresponded very well with the calculated amount of water produced during the esterification. The content of lead in the lead salts of the previous sulphuric acid hydrolysis provided a basis for making this calculation, after an allowance had been made for the glutamic acid removed in the case of Hydrolysis B.

The ester hydrochlorides taken up in dry chloroform. The syrup was taken up in dry chloroform, in which the ester hydrochlorides dissolved readily. A little ammonium chloride, which had been retained in solution by the alcohol, was filtered off and washed with chloroform. The total amount of ammonium chloride formed from the free hydrochloric acid was about 250 g.

Liberation of the esters from their hydrochlorides. The chloroform solution of the ester hydrochlorides occupied a volume of 1000 to 1500 cc. 300 to 350 g. anhydrous baryta were added in successive portions of about 30 g., and the mixture well shaken after each addition. After all the baryta had been added, the mixture was allowed to stand for two minutes, by which time the temperature at the bottom where the solid material had settled had risen to about 35°. A temperature of about 30° throughout the whole fluid resulted on further shaking. The baryta by this time had become granular, and settled instantly the shaking was discontinued. The mixture was cooled under the tap, and a thermometer placed so that its bulb was covered by the solid material at the

bottom. After standing two or three minutes, a rise of 2° was recorded. The mixture was shaken again, cooled, and the test repeated, but no further increase of temperature resulted. It was concluded therefore that the reactions were complete. It may be found sometimes, however, that the clear fluid contains a little chloride at this stage. In this case the mixture should be allowed to stand, with an occasional shake, until no chloride remains. In one experiment the addition of a little more anhydrous baryta was necessary before the last traces of chloride could be removed. The solid material was filtered off by suction and washed thoroughly with dry chloroform. The chloroform was removed from the filtrate by evaporation at 40° and 15 mm., and the esters shaken with anhydrous ether. Practically the whole dissolved immediately, only a very small quantity of syrup which collected on the sides of the flask remaining insoluble. The latter was found to contain substances precipitated by phosphotungstic acid after hydrolysis. The esters weighed 235.9 g.

Recovery of a second portion of esters. The baryta residue obtained when the esters were liberated from their hydrochlorides contained 9.6 g. N. This was suspended in water, and the barium all converted into sulphate at 40° by adding a slight excess of dilute sulphuric acid. After heating for some time on the water-bath, the barium sulphate was removed and washed. The solution was steamed with litharge in the manner already described, and 116 g. dry lead salts obtained. Esterifying these lead salts as before, using equivalent amounts of the various reagents, only 25.6 g. of esters resulted.

The two lots of esters, together amounting to 261.5 g., were combined and distilled in the apparatus described by Fischer and Harries [1902]. For reasons which will be explained later (p. 393) it is regretted that these two quantities of esters were not investigated separately.

Fractional distillation of the 261.5 g. esters.

Fraction	Temp. of bath $^{\circ}$ C.	Temp. of vapour $^{\circ}$ C.	Pressure mm.	Weight of fraction, g.
I.	Up to 88	Up to 65	11	28.07
II	88 to 110	65 to 84	10	73.71
III	110 to 132	84 to 94	10	20.00
IV	152 to 165	100 to 125	10	13.57
Distillation residue	—	—	—	82.03
In liquid air condenser	—	—	—	10.45
Total				227.83

Fraction IV did not commence to distil over until the temperature of the bath was 152° and of the vapour 100° . A very slow current of carbon dioxide, too small to increase the pressure, was passed through the liquid during the distillation. The fractions were thus assisted in their passage to the condenser, and air was excluded. An accident to the Dewar's flask containing the liquid air supply just before the reduction of pressure was required prevented the usual fractions at 0.5 mm. from being obtained. The distillation was continued

at 10 mm. as described, and a very satisfactory separation of the fractions containing glycine, alanine, valine, leucines and proline was effected.

Phenylalanine. Fraction IV was treated with water and extracted with ether in the usual way, and the ether-soluble ester evaporated to dryness with hydrochloric acid. The crystals obtained were dissolved in water, decolorised with animal charcoal, and recrystallised from hydrochloric acid. The specimen was washed thoroughly with saturated aqueous hydrochloric acid, and the free acid removed in a desiccator over potash. This fraction of the phenylalanine hydrochloride, dried completely at 100°, weighed 1.36 g. It contained 7.07 % N (Kjeldahl).

The distillation residue was treated in exactly the same way as Fraction IV, yielding 11.71 g. of the dry recrystallised hydrochloride. This contained 6.87 % N; calculated for phenylalanine hydrochloride 6.95 % N.

The total quantity of hydrochloride separated was 13.07 g. equal to 10.70 g. phenylalanine.

The liquids from which these separations of the hydrochloride had been made were combined, and an attempt made to obtain a further crop of the hydrochloride. A little more was separated, but its content of nitrogen was 0.45 %, too high. It was returned therefore, and the amino acids liberated by quantitatively removing the free as well as the combined hydrochloric acid. A total weight of 7.89 g. dry substance resulted, from which 0.25 g. tyrosine was separated, leaving 7.64 g. in which nothing was satisfactorily identified.

Glycine and alanine. The esters of Fraction I, obtained up to a temperature (vapour) 5° higher than usual, were hydrolysed by boiling with water, and the solution evaporated to dryness. After extracting proline in the usual way, the amino acids, dried at 100°, weighed 13.28 g. By fractional crystallisation from water, one fraction was obtained which melted at 250°, and contained 13.05 % N. This was sub-fractionated, but no portion contained a much higher percentage of N, and no alanine could be separated. The other original fractions gave much higher melting points, and judging by their nitrogen content consisted of mixtures of valine and leucine.

The mother liquor on evaporation yielded 6.58 g. dry amino acids, which melted at 239°, and contained 16.03 % N. This was examined for glycine by the picrate method of Levene and van Slyke [1912], and 2.38 g. glycine picrate melting at 200°, and decomposing at 202°, was separated. The picric acid was removed from the filtrate by means of *N* sulphuric acid and ether and the sulphuric acid by baryta. The amino acids recovered, dried at 100° C., weighed 4.77 g. This material was crystallised from water, leaving a small mother liquor which on evaporation yielded 1.45 g. dry substance melting at 238°, from which a further 0.756 g. glycine picrate with the correct melting point was separated. The two portions of glycine picrate, having identical melting points, were mixed, and the amino nitrogen in a portion weighed out from the whole quantity was determined by the nitrous acid method: found 7.33 % (corrected); calculated for glycine picrate 7.39 %.

The total quantity of the picrate thus separated from the amino acids of Fraction I was 3.136 g., which corresponds to 1.24 g. glycine.

The contents of the liquid air condenser, obtained when the esters were distilled, were freed from ether and hydrolysed by boiling with water. On evaporation 0.36 g. dry substance was obtained which melted at 239°, and contained 16.07 % N. This yielded 0.1 g. glycine picrate with the correct melting point, showing that losses were falling upon glycine ester during the distillation. The ether distilled from the ethereal solution of the esters before they were fractionated yielded by similar treatment 0.17 g. dry substance, from which no trace of glycine picrate could be separated.

The substance separated by crystallisation from the 4.77 g. amino acids recovered after removing the first lot of glycine picrate was submitted to analysis:

	Found	Calculated for alanine
C	40.82 %	40.45 %
H	7.97	7.86

It was concluded therefore that the 6.58 g. portion consisted of a mixture of 1.24 g. glycine and 5.34 g. alanine. This statement is supported by the fact that a mixture of glycine and alanine in these proportions would contain 16.15 % N, whereas 16.03 % was actually found.

Glycine from pure caseinogen. The examination of the first fraction obtained by distilling the esters prepared from the hydrolysis products of "Hammersten casein" by the method advocated in these pages revealed the presence of glycine. Evidence had been given which showed that losses of glycine occurred during the distillation owing to the low boiling point of its ester. The products of pure caseinogen therefore very probably contain considerably more than the 0.45 % isolated as the picrate. The specimen of picrate was excellent in quality. The 0.5 g. triglycylglycine ester separated in the previous hydrolysis (p. 380) provides confirmatory evidence.

If the absolute purity of "Hammersten casein" be doubted, it would be reasonable to suspect contamination with small quantities of the other protein constituents of milk. So far as I can ascertain, no glycine has been found amongst the hydrolytic products of lactalbumin, and the ester hydrolysis of lactoglobulin has not been done. A yield of 3.5 % glycine, however, has been obtained from serum globulin, which closely resembles lactoglobulin. Assuming lactoglobulin to contain 3.5 % glycine, and the 0.45 % glycine isolated from the products of caseinogen to be derived from lactoglobulin as impurity, the presence of 13 % of the latter in the caseinogen must be admitted. Emmerling [1888] states that milk contains not more than about 0.005 % lactoglobulin. Crowther and Raistrick [1916] however isolated 0.03 %, which is equivalent in quantity to only about 1 % of the caseinogen. The glycine separated therefore cannot be attributed to protein impurity.

Valine and leucines. The esters of Fractions II and III were hydrolysed in the usual way by boiling with water, and the solutions combined. The dry

amino acids obtained on evaporation were thoroughly extracted with absolute alcohol to dissolve proline, and dried at 100°. The following results were obtained on analysis: C 53.28, 53.22 %; H 9.67, 9.52 %; N (Dumas) 11.27, 11.28 %.

From these figures the proportion of leucine isomers to valine in the mixture was calculated as follows:

$$\frac{53.28 - 51.24}{54.92 - 51.24} \times 100 = 55.4 \% \text{ leucine isomers.}$$

$$\frac{11.27 - 10.68}{11.97 - 10.68} \times 100 = 45.7 \% \text{ valine.}$$

It will not be far from the mark therefore to conclude that the mixture consisted of 55 % leucine isomers and 45 % valine. To verify this further the lead method of Levene and van Slyke [1909] for the separation of these substances was employed. The lead salt of the leucines which separated was found to contain 44.64 % Pb. Calculated for lead leucine, 44.34 % Pb.

The leucine and valine were recovered from the lead precipitate and from the filtrate respectively, and their content of nitrogen determined.

Nitrogen in the leucine isomers:

0.1995 g.; (Kjeldahl) NH_3 neutralising 15.4 cc. $\text{N}/10$ acid, 10.80 % N. Calculated for leucine, 10.68 %.

Nitrogen in the valine:

0.2292 g.; (Dumas) 22.6 cc. N at 14.5° and 768 mm., 11.90 % N. Calculated for valine, 11.97 %.

The dry amino acids insoluble in alcohol obtained from the ester Fractions II and III, together with the small portions from Fraction I which showed a percentage of N between that of leucine and valine, amounted to 50.85 g. 55 % equals 27.97 g. leucines, leaving 22.88 g. valine.

Proline. The united alcoholic extracts of the dry matter obtained from ester Fractions I, II and III, already in such condition that the amino acids they contained would completely dissolve when well stirred in cold alcohol, were freed from alcohol, and the content of proline calculated from the non-amino nitrogen content according to van Slyke [1911]. The following results were obtained:

Total N	3.44 g.
Amino N	0.761
Proline N	2.679

This amount of proline N corresponds to 22.005 g. proline.

The material was dried to constant weight in the steam-oven, and weighed 28.05 g. It contained therefore 12.26 % total N, which is in accord with the experience of Osborne and Guest [1911]. A 2 g. portion was treated with phenyl isocyanate, and a moderate yield of the proline derivative was obtained. This was converted into the hydantoin, which melted at 143°.

Assuming all the non-amino N to be in the form of proline, 6.05 g. amino acids of the straight chain type were also present in the alcoholic extracts.

Aspartic acid. The esters of Fraction IV, from which phenylalanine ester had been extracted, were hydrolysed by boiling with baryta. A small quantity of barium aspartate separated which yielded 0.48 g. aspartic acid. This was converted into the copper salt which crystallised from plenty of water in the form of light blue needles. The barium was removed quantitatively from the filtrate from the barium aspartate, and the solution evaporated to dryness. After drying at 100°, this material weighed 3.71 g. By means of the titration method of Osborne and Liddle [1910] the presence of a further 0.51 g. aspartic acid was shown. The liquid which had been titrated was concentrated, and a small crystal fraction separated from dilute alcohol, melting at 245°. This substance was found to contain 39.39 % C, 7.82 % H, and 11.25 % N. An attempt to make a β -naphthalenesulphonic derivative gave no satisfactory result. The amino acids obtained from ester Fraction IV, which were not identified, weighed 3.2 g.

THE DISTILLATION RESIDUE.

The esters remaining in aqueous solution after extracting phenylalanine ester with ether from the distillation residue were hydrolysed by boiling with baryta for several hours. The deposit which formed on long standing contained no aspartic acid. The cold filtrate was made neutral to litmus with sulphuric acid as a preliminary to the complete removal of barium. The precipitate which resulted was flocculent and had not the appearance of barium sulphate. It was filtered off, shaken with cold water, decanted several times, and washed many times on the filter with cold water. On investigation this precipitate was found to contain 3.5 g. amino acid material consisting of two unidentified portions, one of which was precipitated by phosphotungstic acid, and aspartic acid which was separated easily and of high purity.

The flocculent precipitate was extracted with successive quantities of boiling dilute sulphuric acid until the extract came through colourless. The solution was reduced in volume, and while at the boiling point sufficient baryta was added to reduce the content of sulphuric acid to 5 %. After heating on the water-bath and filtering, the fluid was cooled and treated with 25 % phosphotungstic acid in 5 % sulphuric acid solution until precipitation was complete. The precipitate was decomposed in the usual way, and yielded 0.64 g. material brown in colour, not hygroscopic, and strongly acid to litmus. The filtrate was freed from phosphotungstic and sulphuric acids, and evaporated to dryness on the water-bath. The dry material weighing 2.88 g. was extracted and washed with a small quantity of ice cold water, yielding a very soluble slightly coloured portion, and a perfectly white insoluble part, weighing 0.85 g. which proved to be aspartic acid. It was found to contain 36.13 % C and 5.27 % H; calculated for aspartic acid, 36.09 % C and 5.26 % H.

A portion was converted into the copper salt, which separated from a dilute aqueous solution in the characteristic form.

The fraction soluble in the ice cold water was converted into the copper

salt, which would not crystallise from water. It dried to a dark blue brittle material, pale blue when powdered, and insoluble in alcohol. Its identity was not established. The analytical figures, given below, are not unlike those obtained for some of the copper salt fractions described on page 389.

Copper	25.86 %
Nitrogen	8.0
Carbon	33.14
Hydrogen	4.14
Water lost at 125°	3.84

From the foregoing it appears that considerable losses of aspartic acid may occur when barium is removed quantitatively as the sulphate from amino acid solutions containing this substance. In all probability the considerable amount of nitrogen rigidly retained by the large barium sulphate precipitate removed in the early stages of the previous sulphuric acid hydrolysis (p. 379) is partially due to aspartic acid.

The solution from which the flocculent barium precipitate had been removed was made to contain 5 % sulphuric acid, and 25 % phosphotungstic acid dissolved in 5 % sulphuric acid added until the supernatant fluid had lost its brownish colour. This precipitate was removed, and a second fraction obtained by continuing the treatment with phosphotungstic acid until the precipitation was complete. These precipitates were decomposed in the ordinary way. The first one yielded 9.18 g. brown material acid in reaction to litmus. The second gave 10.1 g. amino acids alkaline to litmus from which 3.31 g. lysine picrate was obtained by adding an alcoholic solution of picric acid.

The amino acids not precipitated by phosphotungstic acid. On working up the filtrate from the phosphotungstic acid precipitates, a syrup weighing 24.7 g. very acid to litmus was obtained. This was extracted with alcohol. The insoluble part yielded nothing crystalline from water.

A concentrated aqueous solution of the extract gave two small successive fractions on long standing in a desiccator over sulphuric acid. The first, nodular in form, weighed 0.49 g., contained 62.54 % C, 6.93 % H, and 8.74 % N. Calculated for phenylalanine: 65.5 % C, 6.66 % H, and 8.48 % N. The odour of phenylacetaldehyde developed on warming a portion with dilute sulphuric acid and potassium bichromate.

In order to obtain dry matter suitable for analysis so that a general idea of the composition could be formed, the syrupy materials and their fractions were converted into copper salts, and the copper salts fractionated as described below.

Syrupy material insoluble in alcohol:

- A. Copper salt crystallised from water.
- B. Copper salt precipitated from concentrated aqueous solution by alcohol.
- C. Copper salt, derived from material more soluble in water than that which gave copper salts A and B, precipitated from strong aqueous solution by alcohol.

Syrupy material dissolved by alcohol:

D. Copper salt, made from material which had crystallised from water as already described, precipitated by alcohol out of strong aqueous solution.

E. Copper salt precipitated from strong aqueous solution by alcohol.

F. Copper salt soluble in alcohol, precipitated by ether from alcoholic solution.

The analytical data for these copper salts are given in the following table:

	A	B	C	D	E	F
Carbon	—	32.90	31.54	—	35.15	39.79
Hydrogen	—	4.90	4.88	—	4.64	5.35
Nitrogen	8.0	8.51	8.80	—	8.0	8.83
Copper	26.11	26.18	28.74	27.57	23.63	18.90
Amino nitrogen	—	—	6.84	—	3.45	1.34

These copper salts swelled up considerably during ignition, and gave very disagreeable smells. The first four were blue when moist, and light blue in colour when dry. E gave a deep blue water solution, and a pale greenish blue dry salt precipitated by alcohol. F gave the deepest possible blue alcoholic solution, and a very pale blue dry salt when precipitated by ether.

Copper salt B. The copper was removed from this salt, and the aqueous solution concentrated to the syrupy stage. A very small quantity of nodular crystals separated on long standing, which were removed and recrystallised. The dried material weighed 0.18 g., softened at 180°, completely melted at 215°, gave a strong pyrrole reaction when warmed with zinc dust, and contained 44.42 % C, 6.78 % H, and 11.08 % N. These figures correspond approximately to a calculated formula of $C_5H_9O_3N$. The specimen was too small for conclusive identification.

Copper salt C. The copper was removed and 0.0493 g. crystals resembling glutamic acid were obtained from the concentrated aqueous solution. M.P. 208°. Contained 9.58 % N, calculated for glutamic acid 9.52 % N.

Copper salt E. Gave the pyrrole reaction on heating with zinc dust, but not so strongly as copper salt F.

Copper salt F. Weight = 2.5 g. Strong pyrrole reaction. Approximate formula for the free substance calculated from the analytical figures was $C_5H_9O_3N$. Impurity represented by the amino nitrogen (15.8 % of the total N) probably present. The properties and conditions strongly favour the view that the main constituent was the copper salt of pyrrolidonecarboxylic acid, the copper content differing by only 1 % from theory.

The syrupy material. Discussion.

No way was found for isolating a chemical individual from this viscous matter. Salt F was probably the impure compound of pyrrolidonecarboxylic acid. The ratio N : O in salt E was practically 1 : 3.56, and about 1 : 3 in B and C. A trace of glutamic acid was isolated from C. The composition of the free acids calculated from the data given for the copper salts resembles that of the "gummy substance" obtained by the lime-alcohol method for dibasic

acids [Foreman, 1914]. There can be no doubt that the same material is represented. The total weight of unidentified matter obtained from the distillation residue, omitting diamino acids, was 43.7 g. A considerable quantity, probably of peptide nature, was acid to litmus although precipitated by the ordinary phosphotungstic method for separating diamino acids.

THE UNESTERIFIED RESIDUE.

The baryta residue obtained when the second lot of esters were liberated from their hydrochlorides, completely freed from esters by washing with chloroform, was suspended in water, and the baryta all neutralised with sulphuric acid at 40–50°. After heating on the water-bath, the barium sulphate was removed and washed. The liquid was reduced in volume, sulphuric acid added until 5 % was present, and treated with 25 % phosphotungstic acid in 5 % sulphuric acid solution until precipitation was completed. The precipitate decomposed in the usual way yielded 12.96 g. dry matter. The filtrate was freed from phosphotungstic and sulphuric acids, and as much of the free hydrochloric acid as possible removed on the water-bath. The syrup which resulted was taken up in water, made up to 200 cc., and by estimating in aliquot portions, the whole solution was found to contain 1.568 g. total N, 1.043 g. amino N (van Slyke), and 0.042 g. N as NH_3 . The hydrochloric acid remaining was quantitatively removed and the liquid again treated with phosphotungstic acid as before. A small gummy precipitate was obtained, and the whole of the colour disappeared from the solution. The reagents were completely removed, the liquid evaporated, and made up to 200 cc. The amino acid content was estimated by evaporating an aliquot and drying the residue to constant weight at 100°; 10 cc. gave 0.503 g. Allowing for the portions removed in the nitrogen estimations, the unesterified residue therefore contained only 10.06 g. amino acids not precipitated by phosphotungstic acid.

The 0.5 g. obtained from the aliquot was returned to the main solution, and the volume reduced to about 50 cc. on the water-bath. 2.76 g. tyrosine crystallised.

Serine. The filtrate from the tyrosine was evaporated down to 10–15 cc. On standing over night, crystals in the form of thin hard transparent plates separated. This substance was recrystallised from water after removing a trace of colour from the solution by animal charcoal, and yielded crystals of the same character, which weighed 0.998 g. M.P. 231°, with decomposition. Analysis gave the following results:

	Found	Calculated for serine
	%	%
C	34.49	34.29
H	6.87	6.67
N	13.48	13.33

A syrup obtained from the remaining material. An attempt was made to obtain a second crop of serine, but when the concentration was carried further than before, the solution became viscous, and although crystals nodular in form appeared on standing, they could not be separated by suction. A very small quantity from which most of the liquid had been removed by suction gave a copper salt, deep blue in colour, insoluble in alcohol, which contained 19.56 % Cu. The viscous matter resembled cheese in consistency after standing a long time *in vacuo* over sulphuric acid. After treatment with a little cold water, a few large hard crystals were picked out, washed, and recrystallised. This specimen weighed only 0.2 g., and melted at 213°.

Material separated from the syrup by means of basic lead acetate and alcohol. A satisfactory precipitate was obtained by means of alcohol after adding basic lead acetate solution to the aqueous solution of the syrup. The addition of alcohol was continued until the separation was complete. The lead was removed from the washed precipitate, and the resulting solution reduced to very small volume. Large white spherical nodules separated from the viscous solution on standing over night. These were removed, and a second fraction of the same type obtained in the same way. The fractions were recrystallised from water.—No. 1 gave 0.123 g., M.P. 182°; No. 2, 0.05 g., M.P. 181°. A trace heated with zinc dust gave a very strong pyrrole reaction. The material, which appeared to be definite in character, gave the following results on analysis:

C	39.97 %
H	6.33
Amino N	9.94

Although the percentages do not differ widely from those of glutamic acid, further work showed that the substance would not give a hydrochloride insoluble in saturated aqueous hydrochloric acid. The total weight of material precipitated by basic lead acetate and alcohol was 2.23 g.

The amino acids not precipitated as lead compounds. The filtrate from the lead precipitate was freed from lead. Spherical nodules separated from the concentrated aqueous solution on standing. The mother liquor on further concentration yielded crystals of the same kind; M.P. 263° after recrystallisation from water. These gave a strong pyrrole reaction. The quantity obtained was too small for analysis. (Hydroxyproline melts at 270°.)

Discussion.

The unesterified residue contained only 10.06 g. monamino acids. The identifiable part consisted of tyrosine and serine, which are both hydroxy acids. The remainder was syrupy. No traces of known amino acids containing two oxygen atoms in the molecule were found. Further work may show that only the lead salts of hydroxy acids resist esterification. The two small specimens derived from the lead-alcohol precipitate seemed to be definite in character, and the analytical data are not inconsistent with the view held that the substance was probably a hydroxy acid.

THE YIELD OF MONAMINO ACIDS.

The amount of each monamino acid separated in the course of the foregoing investigations will be found in the following table:

Amino acid	Reference page	Weight in g.		%
Glycine	385	1.24		
"	385	0.04	1.28	0.45
Alanine	385		5.34	1.85
Valine	386		22.88	7.93
Leucines	386		27.97	9.70
Proline	386		22.01	7.63
Phenylalanine	384	10.70		
"	388	0.49	11.19	3.88
Serine	390		1.0	0.35
Aspartic acid	387	0.48		
"	387	0.51		
"	387	0.85	1.84	0.64
Glutamic acid	381		45.87	15.91

Discussion.

With the exception of aspartic acid and serine, the percentages are all appreciably higher than those given by Osborne and Guest [1911] in their compounded table which shows the highest reliable figures for each amino acid obtained from caseinogen up to that date. The yield of aspartic acid is much lower than that obtained in Hydrolysis *A* (p. 379), owing to the fact that no liquid air was available when the esters were distilled.

THE NEW METHOD FOR PREPARING ESTERS.

The older methods for preparing the esters of the amino acids leave much to be desired from the point of view of yield. This is chiefly due to the fact that water is added to the ester hydrochlorides and the esters are subjected to the saponifying influence of aqueous alkali during their liberation and extraction. Levene and van Slyke [1909] brought about a considerable reduction in the content of free alkali in the aqueous solution by substituting anhydrous baryta for the soda or potash previously employed and better yields resulted. The esters however are exposed, in their method, to aqueous baryta during the extraction which occupies three-quarters of an hour, and a certain amount of saponification occurs. Their original syrup contains a considerable amount of free hydrochloric acid as well as the ester hydrochlorides, and on adding the baryta a large amount of heat of neutralisation as well as further water develop within a thick mass which in a few minutes becomes semi-liquid and alkaline. In spite of the cooling by means of a freezing mixture, local overheating in such material appears likely. The mass eventually breaks up into granules, remaining in this condition until the operations are concluded. A complete extraction of such material, for the greater part of the time in a wet condition, by stirring with successive additions of ether, seems highly improbable. Saponification and incomplete extraction probably account for the

results obtained by the authors, expressed by them as follows: "Extraction as described...yields a second smaller crop of esters, and by repeating the process a third and even a fourth crop in decreasing yields may be obtained."

Esterification and saponification are both influenced in degree by the amount of water present. The new method for the preparation of esters was designed to eliminate the adverse effect of water upon the yield. As the extraction of esters from large bulks of moist material with ether is probably very incomplete, the esters are liberated in a large volume of a more suitable solvent which protects them from saponification, and from which the residue can be filtered and washed. The following statements will explain the procedures adopted:

(1) The water of the original solution of amino acids is eliminated by obtaining the amino acids in the form of dry lead salts.

(2) In the esterification the reverse reaction is prevented by a large excess of alcohol and the favourable influence of the lead chloride formed.

(3) Excess of free hydrochloric acid is removed from the ester hydrochloride solution without the formation of water.

(4) In the absence of free hydrochloric acid, the water of esterification can be removed with the alcohol from the alcoholic solution of the ester hydrochlorides.

(5) The ester hydrochlorides readily dissolve in chloroform, which can be very easily dehydrated by means of anhydrous baryta.

(6) The amount of water produced by the action of the baryta upon the hydrochloric acid part of the ester hydrochloride molecules is small, as two molecules of hydrochloride are required to give one of water. Water is very sparingly soluble in chloroform (100 cc. dissolve 0.152 cc. water at 22° [Herz, 1898]), and the mass of anhydrous baryta relative to that of the water produced is very large. The water is therefore rapidly taken up. The esters on the other hand are very readily soluble in chloroform, and consequently are removed from the spheres of action immediately. There is practically no exposure of the esters to the influence of aqueous baryta, and saponification is prevented. The increase in temperature which occurs produces little if any ill effect upon the yield, and cooling by means of freezing mixtures is unnecessary. The esters are completely separated from the residue by the simple method of filtering and washing.

The efficiency of the method. The second crop of esters obtained in Hydrolysis *B* weighed only 25.6 g. and probably included a little ether. The opinion was formed that this quantity consisted of the esters of dibasic material, and was practically free from the monobasic acids whose esters appear in the first three fractions of the ester distillation. It is regretted that this portion was not investigated separately. The opinion was based upon the following facts. In Hydrolysis *A*, the yield of glycine, alanine, valine and leucines, obtained from a single ester preparation, together amounted to 18.2 %, and the proline was 6.98 %. The corresponding figures for Hydro-

lysis *B*, in which the second crop of 25.6 g. esters was combined with the first before distillation, were 19.93 and 7.63 respectively. As yield was the chief object of Hydrolysis *B*, the experiments were more carefully conducted and the caseinogen was hydrolysed by means of hydrochloric acid, whereas in Hydrolysis *A* sulphuric acid was employed. The yields are generally not so good when sulphuric acid is used, probably because the hydrolysis is not carried so near to completion and loss of amino acids occurs on removing the large barium sulphate precipitate (p. 379).

In another hydrochloric acid hydrolysis of a kilo of "Hammersten casein," equal to 901.3 g. of the pure substance, one preparation of esters by the new method was made, and the first three ester fractions were distilled in the same manner and up to the same temperature as in Hydrolysis *B*. After extracting the amino acids of these three fractions with hot alcohol, 163.03 g. of a mixture of glycine, alanine, valine and leucines resulted, which corresponds to a total yield of 18.1 %. The small quantities of these amino acids which separate in the ordinary way from the proline extract before it will enter completely into solution in cold alcohol are not included in the calculation. The freedom of the unesterified residue of Hydrolysis *B* from amino acids of the leucine type supports the view that the small second crop of esters contained practically none of these substances.

The very large distillation residue obtained, and the very small quantity of monamino acids found in the unesterified residue, are facts which also point to the high efficiency of the new ester method.

The fractionation of the esters. In distilling the esters of Hydrolysis *B*, four fractions were obtained without the aid of liquid air. After Fraction III was complete, a rise of 6° "vapour" temperature and 20° in the "bath" occurred before further esters commenced to distil over. Fraction IV yielded only 1.36 g. phenylalanine hydrochloride, while nearly nine times as much was obtained quite as easily from the distillation residue. The other material derived from Fraction IV was apparently free from amino acids of the leucine type, and, so far as the estimations were concerned, no advantage was gained by distilling this fraction. Now that the dibasic acids can be easily and completely removed before esterification, their estimations do not depend upon the working up of the higher boiling esters, a method which usually gave very poor yields, owing to the large amount of decomposition. If serine is present in the distillation residue, it will probably crystallise quite readily in the absence of dibasic acids, syrupy products and phenylalanine. It appears, therefore, that there is no need to continue the ester distillation further than Fraction III if the work is carried out on the lines suggested, and the esters of the distillation residue will not be subjected to the influence of the higher temperatures.

IMPROVEMENTS SUGGESTED BY THE RESULTS OF LATER EXPERIMENTS.

The foregoing experiments were completed in 1913. Further work showed that the syrupy material found in such large quantity in the distillation residue, as well as to some extent in the unesterified fraction, can be removed from the original hydrolytic mixture by the "lime-alcohol" method for separating dibasic amino acids [Foreman, 1914].

Further investigations on the nature of the new syrups prepared under certain temperature precautions, and on the possibilities of the "lime-alcohol" method for simplifying the mixture of amino acids before esterification, were carried out in the latter part of 1914. The work was suspended early in 1915 owing to war conditions. The experiments will be described in a further paper, and the results compared with those obtained by Dakin [1918] who has since identified a new amino acid, β -hydroxyglutamic acid, in the syrups which the "lime-alcohol" method enabled him to obtain.

The further investigations showed that on working up the alcoholic filtrate from the insoluble calcium salts, the monobasic amino acids can be obtained in the form of a light brown powder, proving that all the viscous matter can be removed by the "lime-alcohol" method. Impure proline was obtained by extracting the powder with alcohol. The ester process however will probably yield purer proline.

The advantages likely to be gained by removing the alcohol-insoluble calcium salts before applying the new method for preparing esters are given in the following summary:

- (1) A single ester preparation will contain the whole of the esters of the monobasic monamino acids, with the possible exception of those of the hydroxy type.
- (2) The esters will fractionate more easily, and the difference between "bath" and "vapour" temperatures will be reduced, thus decreasing the risk of decomposition.
- (3) Substances of syrupy nature will not be present to complicate the issue at almost every stage. The separations will therefore be more complete, and the amino acids yielded in purer condition.
- (4) The residues will be very much simpler in composition, and the amino acids they contain thrown into much greater relief.

COMPOSITION OF CASEINOGEN.

The sum of the percentages of the known amino acids separated from the products of caseinogen by the older methods showed a very considerable deficit. The method for estimating diamino acids gives very satisfactory results, and consequently it is generally believed that the loss falls almost entirely upon the monamino acids during their isolation and estimation. The preparations of esters by the older methods were incomplete, and definite information in regard to the nature of the amino acids making up the de-

iciency could not be gained by examining the residues. The methods of preparing and distilling the esters of Hydrolysis *B* made a better examination of the residues possible. The distillation residue yielded material probably of peptide character (p. 390), indicating that a complete hydrolysis of caseinogen is not effected by boiling for 48 hours with hydrochloric acid, as well as a large amount of syrupy material of unknown constitution. The amount of mon-amino acids in the unesterified residue was very small and consisted of hydroxy acids together with further syrups. Neither of these residues gave any indication that amino acids of the leucine type were present. The syrupy material was afterwards separated directly from the hydrolytic products, together with glutamic and aspartic acids, in the form of alcohol-insoluble calcium salts [Foreman, 1914], and as much as 37.88 % of the caseinogen was accounted for by this method.

Our present knowledge of the quantitative proportions of the products obtained by the acid hydrolysis of caseinogen, including the results of these investigations, may be summarised as shown in the accompanying table. The percentage of lysine was calculated from the figures given by van Slyke [1913] for his estimation of this substance by the Kossel method. The figures for the other amino acids represent the highest reliable results as selected by Osborne and Guest [1911] in their review of the information then available. A total of 67.85 % of caseinogen was accounted for by them in this manner, including the figures for elementary sulphur and phosphorus.

Results of Hydrolysis <i>B</i> (1913)	Glycine	0.45 %
			Alanine	1.85
			Valine	7.93
			Leucines	9.7
			Proline	7.63
			Phenylalanine	3.88
Results obtained by the method for separating dibasic amino acids [Foreman, 1914]			Glutaminic acid	21.77
			Aspartic acid	1.77
			New Syrups	14.34
Van Slyke	Lysine	7.62
Results selected by Osborne and Guest	...		Histidine	2.5
			Arginine	3.81
			Tryptophan	1.5
			Serine	0.5
			Cystine	—
			Tyrosine	4.5
			Hydroxyproline	0.23
			Diaminotrihydroxydodecanic acid	0.75
			Ammonia	1.61
			Sulphur	0.76
			Phosphorus	0.85
								93.95
Obtained from Hydrolysis <i>B</i> , probably of peptide nature (p. 390)	3.41
								97.36

The products include the water of hydrolysis, and the total should be greater than 100. The application of the new ester method after removing the

dibasic material will very probably throw considerable further light upon the composition of caseinogen, and further investigation is proceeding on these lines.

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XXXVII. ON AMINO-ACIDS. PART II. HYDROXYGLUTAMIC ACID.

BY HENRY DRYSDALE DAKIN.

REPORT TO THE MEDICAL RESEARCH COMMITTEE.

(Received November 7th, 1919.)

THE object of the following communication is to record the results of experiments made in continuation of work already published in this *Journal* [Dakin, 1918]. In the previous communication it was shown that the products of hydrolysis of a protein, *e.g.* caseinogen, might be extracted with partially miscible solvents, such as butyl alcohol, so that the feebly ionised monamino-acids and proline were substantially removed¹, while the strong acids and bases remained behind. Evidence was presented of the occurrence among the acids, in addition to glutamic and aspartic acids, of a new amino-acid which was regarded as β -hydroxyglutamic acid from a study of its composition and reactions. The present paper contains the results of the further study of this acid together with experiments on its synthesis and that of allied substances. For convenience, the subject-matter is divided into the following sections:

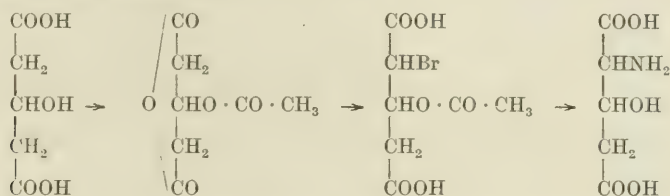
- I. On the synthesis of inactive β -hydroxyglutamic acid and allied substances.
- II. The *p*-nitrophenylosazones of malic semi-aldehyde and tartronic semi-aldehyde.
- III. Optical activity of natural β -hydroxyglutamic acid.
- IV. Alkaloid salts of β -hydroxyglutamic acid and other amino-acids.
- V. Identification of β -hydroxyglutamic acid among the products of hydrolysis of glutenin and gliadin.
- VI. The fate of β -hydroxyglutamic acid in the diabetic organism.

I. ON THE SYNTHESIS OF INACTIVE β -HYDROXYGLUTAMIC ACID AND ALLIED SUBSTANCES.

At first sight it appeared that the synthesis of inactive β -hydroxyglutamic acid would be a relatively simple problem capable of solution by many methods,

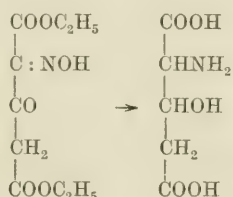
¹ It is perhaps worth recording the fact that if the amino-acid aqueous solution is saturated with potassium carbonate and then extracted with ordinary ethyl alcohol, the monamino-acids are found in the alcoholic extract as potassium carbamates and may be precipitated by acetic acid.

but, at any rate in the writer's experience, this has not proved to be the case. The first method that was tried consisted in brominating the acetyl derivative of β -hydroxyglutaric anhydride described by Blaise [1903] and then acting on the product with ammonia:



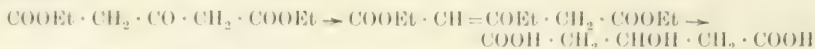
The acetyl derivative is obtained by the action of acetic anhydride on β -hydroxyglutaric acid, prepared by the reduction of acetonedicarboxylic acid. But while the bromination apparently went smoothly, the action of ammonia removed the halogen without introducing an amino-group, probably through the formation of a trimethylene derivative. An analogous reaction was observed by Perkin and Tattersall [1905] in the case of α -bromoglutaric acid.

A more promising method presented itself in the reduction of α -isonitroso-acetonedicarboxylic ester:

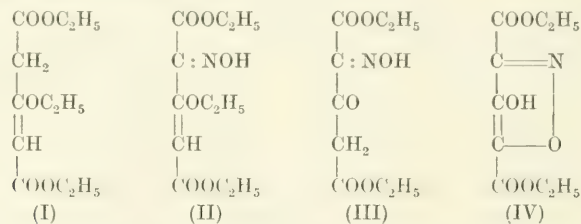


A very large number of experiments were made with this substance, using almost every known reducing agent that had the smallest prospect of success, but the reaction did not proceed smoothly. Using sodium or sodium amalgam in neutral or acid solution, it was an easy matter to reduce the ester and to obtain an acid which, in many ways, resembled the natural hydroxyglutamic acid and had approximately the same composition. But a variable proportion of the product when tested with nitrous acid was found not to be an amino-acid nor was it a hydroxypyrrolidonecarboxylic acid for it did not give an amino-acid on warming with hydrochloric acid. The separation of the products of reaction proved very difficult and has not been satisfactorily accomplished. It appeared that complicated condensations followed the production, as the first stage of the reaction, of α -amino-acetonedicarboxylic ester, such as are known to occur with amino-acetoacetic ester. It therefore seemed desirable to limit the reactivity of the carbonyl group. The introduction of an acetyl group attached to oxygen, by means of acetyl chloride in pyridine solution, proved of no avail. Later the observation was made that ethoxyglutaconic ester, obtained by the action of orthoformic ester on acetonedicarboxylic ester, on reduction with sodium amalgam in the presence of excess of carbon dioxide

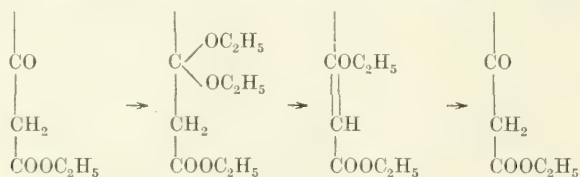
gave β -hydroxyglutaric acid instead of β -ethoxyglutaric acid as might be expected:



It therefore appeared possible that α -isonitroso-acetonedicarboxylic ester might be converted into isonitroso- β -ethoxyglutaconic ester (II) and that the latter might be successfully reduced to hydroxyglutamic acid. Orthoformic ester was found to react vigorously with isonitroso-acetonedicarboxylic ester but the product, which cannot be distilled, sometimes gave results on analysis indicative of the ethoxy ester, but often, after simple manipulations and careful drying, nothing but unchanged nitroso-acetonedicarboxylic ester was obtained. It was clear that the ethoxy group was very easily removed and indeed on reversing the order of the reactions and starting with ethoxyglutaconic ester (I) it was found that nitrous acid could convert it into nitroso-acetonedicarboxylic ester (III) and when present in excess into hydroxy-isoxazoledicarboxylic ethyl ester (IV):



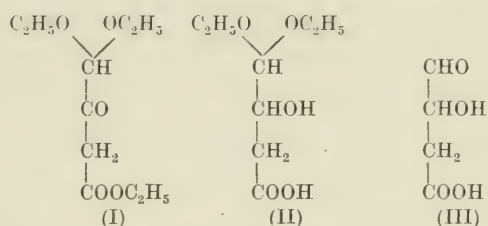
As is well known from Claisen's researches, the initial reaction between a β -ketonic ester and orthoformic ester in the presence of a suitable catalyst involves the formation of a γ -diethoxy ester which usually parts with a molecule of alcohol to give an ethoxy derivative of an olefinic acid, but which in turn may be reconverted into the original ketonic ester by simple hydrolysis:



Accordingly the reaction between orthoformic ester and isonitroso-acetonedicarboxylic ester was effected at low temperatures, and in order to limit decomposition of the product, it was reduced with sodium amalgam after a minimum of manipulation. The results were rather more satisfactory than those obtained by the direct reduction of isonitroso-acetonedicarboxylic ester, and small amounts of an amino-acid were eventually isolated which bore a very close resemblance to natural β -hydroxyglutamic acid. However, the final stage in the preceding synthesis does not proceed as smoothly as might be desired, and as the amino-acid to be finally isolated resembles the natural acid in being extremely soluble and difficult to crystallise or characterise by

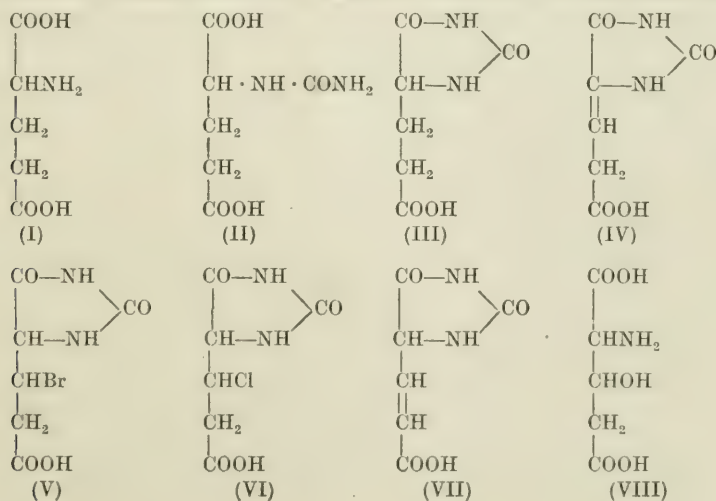
derivatives, and, moreover, readily passes over into a pyrrolidone derivative with loss of water, it appeared desirable to attempt some other mode of synthesis, as a confirmation.

The next method to be tried was based upon Strecker's well-known synthesis of amino-acids from aldehydes by way of the amino-nitriles. For this purpose it was necessary to prepare the hitherto unknown semi-aldehyde of malic acid, *i.e.* β -hydroxy- γ -aldehydobutyric acid. This was successfully accomplished in the following way— γ -diethoxyacetoacetic ester (I) obtained as previously described by Dudley and the writer [1914], was reduced with sodium amalgam to γ -diethoxy- β -hydroxybutyric acid (II). The latter substance, on careful hydrolysis with dilute sulphuric acid, gave malic semi-aldehyde (III):



Unfortunately, the malic semi-aldehyde, when subjected to Strecker's reaction substantially under the same conditions as those used by Fischer in the synthesis of serine from glycollic aldehyde, gave only a negligible trace of an amino-acid. From 20 g. of the aldehyde, only enough amino-acid was obtained to observe its qualitative reactions, which resembled those of the natural acid, and to furnish about 0.2 g. of its silver salt. Attempts to improve the yield were unavailing and the method, which seems often to prove unsatisfactory in the case of hydroxyaldehydes, was abandoned.

More satisfactory results were obtained with a synthesis starting with glutamic acid. Glutamic acid (I) was converted in α -uraminoglutaric acid (II)



by the action of potassium cyanate, and hydantoin-propionic acid (III) was obtained from the latter by warming with hydrochloric acid, as already described [Dakin, 1910]. The action of bromine on hydantoin-propionic acid was then studied. It was found that on warming hydantoin-propionic acid dissolved in excess of glacial acetic acid with a molecular proportion of bromine, the latter quickly disappeared and that much more than half of the bromine was obtained as hydrobromic acid. The products of the reaction reduced permanganate solution freely and could absorb further amounts of bromine, so that it was clear that unsaturated products had been formed. On brominating concentrated solutions in glacial acetic acid, it was found that less of the bromine appeared as hydrobromic acid and a crystalline mixture of substances was separated which appeared to be made up of the unsaturated acid together with its hydrobromic acid addition product. The latter could be readily separated in the form of colourless cubes by repeated crystallisation from hydrobromic acid solution and from its reactions and composition is regarded as hydantoin- β -bromopropionic acid (V). It is reduced by zinc dust to hydantoin-propionic acid and, like most β -bromo-compounds of glutaric acid, is very unstable towards alkali. The unsaturated acid was obtained by boiling the mixture just referred to with zinc dust, separating it as zinc salt and then decomposing the latter with hydrogen sulphide. It is a sulphur yellow substance, reducing permanganate freely, and is convertible into hydantoin- β -bromopropionic acid with hydrobromic acid. It absorbs bromine slowly in the cold when dissolved in acetic acid but rapidly at 100°. This substance, to which is assigned the formula (IV), is somewhat difficult to name satisfactorily but may be regarded as *hydantoin- β . γ -propenylic acid*. Its relation to glutamic acid is clear and the possibility of *cis* and *trans* isomers is also evident. It is not improbable that it is formed by the removal of hydrobromic acid from a bromohydantoin-propionic acid in which the bromine is in the γ -position.

Additional evidence as to the constitution of hydantoin- β -bromopropionic acid and its formation by the addition of hydrobromic acid to hydantoin- β . γ -propenylic acid is furnished by the following facts. In the first place, the yield of this substance is increased by brominating hydantoin-propionic acid in the presence of excess of hydrobromic acid. Secondly, although the hydantoin-propionic acid is strongly optically active, the β -bromo-derivative is entirely inactive although containing an asymmetric carbon atom. This result is readily interpreted if the reaction takes place as indicated, for the intermediate unsaturated acid (IV) has lost its asymmetry. Lastly, if the bromination of hydantoin-propionic acid is carried on in fuming hydrochloric acid solution, hydantoin- β -chloropropionic acid (VI) is readily obtained.

A close analogy to the first stage of the reaction under discussion is furnished by the action of bromine upon *p*-hydroxybenzylhydantoin which was found by Johnson and Hoffman [1912] to give the unsaturated 3,5-dibromo-4-hydroxybenzylhydantoin. Gabriel [1906] also observed a similar

change in the formation of "pyvureidic acid" on brominating hydantoin-acetic acid.

The conversion of hydantoin- β -bromopropionic acid into β -hydroxyglutamic acid, a reaction involving the opening of the hydantoin ring by hydrolysis and replacement of the halogen by hydroxyl, is not as easy as might be wished. On warming the acid with excess of barium hydroxide, a clear solution is at first obtained, but in a few moments an insoluble barium salt separates out. This salt is a mixture which contains progressively less nitrogen as the amount of barium hydroxide and time of heating are increased and is mainly made up of barium salts of acids containing two or possibly three carbon atoms together with barium carbonate. On prolonged boiling with excess of barium hydroxide traces of an amino-acid are formed, but the bulk of the substance undergoes complete decomposition. On boiling with pyridine or other bases, the halogen of hydantoin- β -bromopropionic acid is readily removed, but carbon dioxide is evolved and neutral products result. The instability of the five carbon chain of hydantoin- β -bromopropionic acid towards alkali is not surprising in view of the ease with which β -bromoglutaric acid is known to give vinylacetic acid when warmed with soda [Wislicenus, 1899; Ssamenov, 1899].

Better results were obtained on simple boiling of hydantoin- β -bromopropionic acid with water. Hydrobromic acid is slowly split off, and among other complex products a crystalline substance is easily separated in about 20 % yield, which is an unsaturated acid, isomeric with the hydantoin- β . γ -propenylic acid already described. Like the latter substance, it is a bright sulphur yellow compound and absorbs hydrobromic acid and bromine on warming. This substance appears to be hydantoinacrylic acid as represented by formula (VII). On prolonged treatment of this substance with hot barium hydroxide solution, the hydantoin ring is opened with removal of ammonia and carbon dioxide, while the elements of water are taken up with formation of β -hydroxyglutamic acid. The opening of the hydantoin ring by alkaline hydrolysis is of course a common reaction, as is the formation of a β -hydroxy-acid from an unsaturated acid by treatment with alkali, *e.g.* glutaconic acid gives β -hydroxyglutaric acid and fumaric acid yields malic acid. The yield of amino-acid is disappointing, amounting to about 20 % of the hydantoinacrylic acid taken. Calculated on the glutamic acid used as the starting material, the yield is not more than about 2 %. An alternative method of converting hydantoin- β -bromopropionic acid into hydroxyglutamic acid consisted in hydrolysing the former with fuming hydrochloric or hydrobromic acid in a sealed tube at 130°, and subsequently removing the excess of acid and boiling the product with lime water to replace the halogen by hydroxyl. The results were not much more satisfactory than those just described.

The synthetic inactive β -hydroxyglutamic acid isolated as described in the experimental portion of the paper could not be obtained in large enough amounts to permit of a detailed study, but the following points of resemblance

to the natural optically active acid were noted and no dissimilarities were observed.

The acid is extremely soluble in water and crystallises with great difficulty. It is insoluble in alcohol and most organic solvents. The whole of its nitrogen is in the NH_2 form and can be liberated by nitrous acid in van Slyke's apparatus. On heating to 105° , almost the whole of the nitrogen goes over into the (NH) form, through formation of a hydroxypyrrolidonecarboxylic acid with loss of water, and no longer reacts with nitrous acid. The natural acid shows precisely the same behaviour and the ease with which it parts with water accounts for the lack of a sharp melting point. The copper salt, like that of the natural acid, is very soluble in water, insoluble in alcohol. The acid titrates to litmus as a monobasic acid and forms neutral salts with the alkali metals containing one equivalent of base and alkaline salts containing two equivalents. The *silver salt* is a white granular precipitate almost insoluble in water, contains two equivalents of silver and resembles the salt of the natural acid closely. The acid is saturated and does not promptly reduce permanganate in the cold in alkaline solution.

On treating a few milligrams of the substance with diazobenzenesulphonic acid and then adding sodium hydroxide, a cherry red colour develops (or orange red if the solution is very dilute) which becomes intensely deep on warming¹. If sodium carbonate be used as alkali, the colour tone in the cold is orange red which deepens to cherry red on warming. The natural acid, separated from its crystalline strychnine salt, shows the same reaction. Lastly, on gently warming a few milligrams of the acid dissolved in a drop or two of water with β -naphthol and concentrated sulphuric acid, a brilliantly fluorescent yellowish green solution results². The same reaction is shown by the natural acid and by malic acid.

Since β -hydroxyglutamic acid contains two dissimilar asymmetric carbon atoms it may occur in four active forms and at least two inactive forms. It has not been possible to obtain sufficient of the inactive synthetic acid for resolution into active forms so that the space relationships of both active and synthetic acid await further investigation.

In order to save unnecessary duplication by other workers, it may be of some value to indicate a number of other attempted syntheses of β -hydroxyglutamic acid which were tried without success, though of course it is quite

¹ Most amino-acids on *boiling* with diazobenzenesulphonic acid and caustic soda develop a reddish colour, but the reaction is trivial compared with the above. Rosenthaler [1912] has described an analogous reaction for malic acid.

² In the original description of the natural acid it was stated that it gave a violet red colour with resorcinol and sulphuric acid. This is an error and is apparently connected with the use of silver nitrate in separating the acid. Nitric acid gives a similar reaction, but preparations of the amino-acid containing so little nitric acid that no reaction could be obtained with the brucine test still gave a positive reaction. When no silver nitrate was used in the separation of the acid, the characteristic reaction with resorcinol was not obtained. Possibly it is due to a trace of some oxidation product.

possible that some other of them might be successful if more suitable conditions were found than those employed by the writer:

(a) Bromination of benzoylglutamic acid, with the hope of obtaining a benzoylamino-glutaconic acid by removal of hydrobromic acid.

(b) Reduction of *o*-toluidine-azo-acetonedicarboxylic ethyl ester [cp. Bülow and Göller, 1911].

(c) Reduction of various hydrazine derivatives of acetonedicarboxylic ester.

(d) Condensation of oxymethylenehippuric ester with malonic ester.

(e) Condensation of dichloroacetal with malonic ester followed by reactions analogous to Leuch's synthesis of serine.

(f) Reduction of isonitrosoglutaconic ester.

(g) Reduction of hydroxyisoxazoledicarboxylic ethyl ester.

The isolation of β -hydroxyglutamic acid from the products of hydrolysis of proteins makes it desirable to know more of related hydroxy-amino-dicarboxylic acids. Reference was made in a previous paper [Dakin, 1918] to Skraup's statement as to the occurrence of hydroxyaspartic acid in caseinogen. It was there stated that "As Fischer has pointed out Skraup's experiments are not described in a form capable of repetition and no evidence other than the analysis of a copper salt is adduced to show that the minute amount of substance obtained by Skraup was a dicarboxylic acid. The value of this analysis for determining the basicity of the acid is small, since no carbon or hydrogen determinations were made on the free acid." Almost immediately after Skraup's announcement, Neuberg stated that he had obtained hydroxyaspartic acid in small amount by the action of nitrous acid on diaminosuccinic acid. Erlenmeyer and Bade [1904] state that they synthesised the same substance by reducing oxalylhippuric ester, but no account of the process or product has yet appeared, so far as we have been able to ascertain. The writer has endeavoured to synthesise hydroxyaspartic acid and has found that it is formed by the action of ammonia on barium chloromalate, a reaction which is similar to one partially investigated by Lossen. A detailed account of the acid, which has entirely different properties from those described by Skraup and by Neuberg, will form the subject of a separate communication.

It is a curious fact that the amino-acid gives a so-called abnormal copper salt, resembling iso-serine in this respect, as was shown by Fischer. The presence of a hydroxyl group contiguous to a carboxyl group is undoubtedly responsible for the fact that the copper salt contained three equivalents of copper instead of two. The structural similarity between iso-serine which combines with two equivalents of copper and hydroxyaspartic acid is clear from the following formulae:



The formation of this abnormal copper salt of synthetic hydroxyaspartic acid is clearly of value in further questioning the identity of Skraup's acid which was considered to be a hydroxyaspartic acid largely on the basis of the analysis of a copper salt which was believed to possess a normal composition.

EXPERIMENTAL.

In the following section, the experiments leading to the synthesis of β -hydroxyglutamic acid from hydantoin-propionic acid will be considered first, since they have given the most satisfactory results. The preparation of malic semi-aldehyde and its derivatives will then be described. Only a brief outline of experiments on the reduction of isonitroso-acetonedicarboxylic ester and its condensation products with orthoformic ester will be included since they cannot be regarded as satisfactorily completed. The melting points recorded are all "uncorrected" observations, unless otherwise indicated.

Hydantoin-propionic acid. The preparation and properties of this substance have already been described by the writer [1910]. Certain minor changes in its preparation have proved advantageous as regards the yield. Glutamic acid, 29.4 g., is heated on the water-bath with about 100 cc. of water, and strong sodium hydroxide solution is gradually added until the whole of the acid is dissolved and the reaction of the solution is approximately neutral to litmus. To the solution of mono-sodium glutamate thus obtained 20 g. of freshly prepared potassium cyanate (1.2 mols.) is added and the mixture allowed to evaporate slowly over the water-bath for about an hour. The solution is then made acid to Congo-red with hydrochloric acid and an additional 30 cc. of the concentrated acid is added. The solution is then heated in a covered dish on the water-bath for two or three hours in order to convert the uramino-acid first formed in the alkaline solution into hydantoin-propionic acid. The mixture is next transferred to a capacious flask and evaporated to dryness under diminished pressure. The residue is then extracted with boiling alcohol, thus dissolving the acid and leaving sodium and potassium chlorides undissolved. The alcoholic solution is at once diluted with water and the alcohol removed by evaporation. Hydantoin-propionic acid slowly crystallises out of the concentrated solution in a yield fully equal to the weight of glutamic acid taken. The direct evaporation of the alcoholic solution without dilution with water should be avoided, as in this case an uncrystallisable syrup frequently results, possibly owing to ester formation.

Bromination of hydantoin-propionic acid. The first point to be investigated concerned the proportion of hydrobromic acid formed on acting upon hydantoin-propionic acid (1 mol.) with bromine (1 mol.) in glacial acetic acid solution. The experiments were conducted in small sealed tubes which, after heating at 90° until all the bromine had disappeared, were opened under water, acidified with nitric acid and the hydrobromic acid estimated volumetrically with standard silver nitrate solution. Appropriate blanks were carried out and the necessary corrections made. In experiments employing

0.2 g. of the acid and an equivalent amount of bromine in 1 cc. of glacial acetic acid, it was found that hydrobromic acid was liberated equivalent to from 1.5–1.78 molecular proportions. Slightly higher results were obtained on increasing the dilution with acetic acid and lower results if the solvent were reduced. It was therefore clear that a large proportion of any bromine entering the hydantoin-propionic acid molecule was removed as hydrobromic acid, but, as was subsequently found, the unsaturated products formed can again take up hydrobromic acid under suitable conditions.

On brominating larger quantities of the hydantoin-propionic acid dissolved in six parts of glacial acetic acid, as above described, and then mixing the resulting solution with water and crystallising after evaporation, a light yellow substance was obtained melting indefinitely around 210–220°. Under the microscope it was clearly seen to be a mixture and contained about 35 % carbon, 3 % hydrogen and about 20 % of bromine. This mixture furnished hydantoin- β -bromopropionic acid on repeated crystallisation from hydrobromic acid solution, while on reduction with zinc dust it gave hydantoin- β . γ -propenylic acid, as described later. The yield of crystalline mixture obtained amounted to about half the weight of acid brominated.

Hydantoin- β -bromopropionic acid. The preceding experiments clearly indicated that the bromine derivative of hydantoin-propionic acid was almost surely formed by the secondary addition of hydrobromic acid to an unsaturated intermediate product (hydantoin- β . γ -propenylic acid). It was therefore probable that it could best be prepared in a medium already containing preformed hydrobromic acid. Five parts of aqueous hydrobromic acid (50 %) were tried with satisfactory results, but the best yields were obtained as follows.

Hydantoin-propionic acid, 17.2 g., is boiled with 30 cc. of glacial acetic acid in a strong flask and while the mixture is still fairly hot, 35 cc. of glacial acetic acid previously saturated with hydrobromic acid at 0° are added and the mixture at once cooled under the tap. The acid will now readily dissolve and remain in solution. Bromine, 16 g., is added and the flask is then closed and gently warmed at about 60°–70° on a water-bath. In the course of an hour or two, the whole of the bromine will disappear and the mixture is then allowed to stand in a cool place for a day or two. After a time, a crop of white crystals separates and is removed by filtration, using an asbestos pad on a porcelain funnel. On concentrating the mother liquor under reduced pressure, further crops may be obtained leaving a thick syrup as mother liquor. The crystals are recrystallised from boiling 30 % hydrobromic acid and separate as perfectly white cubes and rhombic prisms melting at 228–230° with effervescence and turning deep brown in colour. If crystallised from water, slight decomposition takes place, giving a yellowish product. The yield amounts to 9–11 g. The substance reacts as a monobasic acid to litmus. On titrating 0.25 g. with *N*/10 sodium hydroxide 10 cc. were required, equivalent to a molecular weight of 250, compared with a calculated value of 251.

Analysis. 0.1253 g.; 0.1334 CO₂; 0.0309 H₂O,
0.20 g.; 0.0221 N (Kjeldahl),
0.10 g.; 0.0753 AgBr.

	C	H	N	Br
Calculated for C ₆ H ₇ O ₄ N ₂ Br	28.7	2.78	11.1	31.8
Found 	29.0	2.74	11.0	32.0

Hydantoin- β -bromopropionic acid is readily soluble in hot water, sparingly in cold water, but dissolves more easily in acetic acid. It is sparingly soluble in ether or chloroform. On ignition, the vapours do not give the pyrrole reaction with a pine splinter, but if the substance is mixed either with sodium carbonate or zinc dust before ignition, an intense reaction is obtained. The whole of the bromine is readily removed on warming for a few minutes with strong sodium hydroxide solution, but it is decidedly more stable towards ammonia. On heating with excess of pyridine at 100°, carbon dioxide is split off together with the bromine, and a neutral product, melting at over 270°, results. Its reaction towards boiling water is described in a subsequent section and the action of barium hydroxide has already been referred to. The neutral salts are not particularly unstable, for a small amount of the acid, digested on the water-bath for half an hour with a suspension of chalk in water, lost only about a third of its bromine.

Hydantoin- β -bromopropionic acid (1 part) was reconverted into hydantoin-propionic acid by warming it on the water-bath with granulated zinc (3 parts) and 50 % acetic acid (10 parts). The solution was evaporated to dryness, dissolved in water and the zinc removed with hydrogen sulphide. On concentrating the filtrate, the hydantoin-propionic acid readily crystallised.

Hydantoin- β - γ -propenylic acid. This acid was separated from the mixture of crystalline substances resulting from the bromination in acetic acid solution of hydantoin-propionic acid, as described in the preceding section. The mixture (5 g.) was boiled for one hour with zinc dust (20 g.) and water (400 cc.). The bulk of the hydantoin- β -bromopropionic acid is completely decomposed by this treatment and the remainder reduced to the very soluble hydantoin-propionic acid, while the unsaturated acid is substantially unaffected. On filtering and concentrating the filtrate to small bulk, a deep yellow zinc salt, moderately soluble in water but insoluble in alcohol, crystallises out. This was filtered off, dissolved in hot water, the zinc removed with hydrogen sulphide and the filtrate again concentrated. About 0.7 g. of a sulphur yellow compound, crystallising in fine hexagonal prisms, was obtained. It is readily soluble in hot water and fairly soluble even in cold water. It melts at 222–223° without darkening and gives a solid sublimate. It gives a strong cherry red colour with diazobenzenesulphonic acid in the presence of sodium hydroxide, thus resembling glutaconic acid, of which it may be regarded as a derivative. On warming with silver nitrate and ammonia, very little or no reduction takes place. On treatment with β -naphthol and excess of concentrated sulphuric acid it gives a clear yellowish green solution in the cold, turning suddenly violet red on gentle warming. The pyrrole reaction is positive. The acid re-

duces permanganate freely in sodium carbonate solution, and absorbs bromine or hydrobromic acid in acetic acid slowly in the cold but readily at about 80°.

Analysis. 0.1598 g.; 0.2468 CO₂; 0.0512 H₂O.

	C	H
Calculated for C ₆ H ₆ O ₄ N ₂ ...	42.3	3.53
Found	42.1	3.55

Hydantoin-β-chloropropionic acid. This compound was obtained by warming in a sealed tube at 95° hydantoin-propionic acid (8.6 g.) dissolved in fuming hydrochloric acid (40 cc.) and bromine (8.0 g.). The whole of the bromine disappeared in less than an hour. When the tube was kept in a cold place for a couple of days about 3 g. of crystals separated, and further small amounts of less pure material were obtained on concentrating the mother liquor under reduced pressure. The substance was purified by recrystallisation from 20 % hydrochloric acid, in which it is readily soluble when hot but sparingly soluble in the cold. It crystallises in rectangular plates which are without colour when pure. On recrystallising from water they become yellowish. The reactions of the substance are identical with those of the corresponding bromine compound already described. Using litmus paper as indicator, the substance reacts as a monobasic acid—0.2 g. required 9.77 cc. of N/10 sodium hydroxide equivalent to a molecular weight of 206—the theoretical value being 206.5.

Analysis. 0.1299 g.; 0.1660 CO₂; 0.0381 H₂O,
0.20 g.; 0.0266 N (Kjeldahl),
0.10 g.; 0.0682 AgCl.

	C	H	N	Cl
Calculated for C ₆ H ₇ O ₄ N ₂ Cl	34.9	3.39	13.5	17.2
Found	34.9	3.26	13.3	16.9

Hydantoinacrylic acid. Hydantoin-β-bromopropionic acid (10 g.) was boiled with water (200 cc.) under a reflux condenser for three or four hours. The solution was then concentrated to about 50 cc. and allowed to crystallise. The yellow crystals thus separated were again boiled for a short time with sufficient fresh distilled water to dissolve them and allowed to crystallise again. On diluting the first mother liquor, again boiling and concentrating to small bulk, a little more of the crystalline product may be obtained. The total yield amounts to 15–20 % of the theoretical. It should be tested for the absence of bromine and if any is found it should be again boiled with water, or alternatively it may be dissolved in excess of warm sodium hydroxide and then precipitated with hydrochloric acid. The substance when pure is sparingly soluble in cold water and only moderately so in hot water. It crystallises in rosettes of sulphur yellow needles melting at 256–258° with effervescence. It is very sparingly soluble in acetic acid, alcohol or ethyl acetate. It does not react with phenylhydrazine acetate but reduces ammoniacal silver solutions on boiling. It does not reduce mercuric chloride. Bromine and hydrobromic acid in acetic acid solution are absorbed slowly when cold, but readily on warming. Diazobenzenesulphonic acid gives a strong cherry red reaction with

a trace of the acid dissolved in sodium hydroxide. The substance reacts as a monobasic acid to litmus—0.1427 g. required 8.8 cc. *N*/10 sodium hydroxide, though the end point was not perfectly sharp. This titration is equivalent to a molecular weight of 162 compared with a calculated value of 170.

Analysis. 0.1405 g.; 0.2179 CO_2 ; 0.0480 H_2O ,
0.1425 g.; 0.0248 *N* (Kjeldahl).

	C	H	N
Calculated for $\text{C}_6\text{H}_6\text{O}_1\text{N}_2$...	42.3	3.53	16.5
Found	42.1	3.81	16.7

β -Hydroxyglutamic acid. Hydantoinacrylic acid (2 g.) was boiled under a reflux condenser with recrystallised barium hydroxide (20 g.) and water (60 cc.). At the top of the condenser a small absorption bulb was placed containing standard sulphuric acid and a little alizarin as indicator. Whenever the contents of the bulb became alkaline owing to absorption of ammonia, 1 cc. more normal acid was added. In this way it is possible to follow the course of the hydrolysis and to stop the heating after ammonia has been liberated equivalent to half the nitrogen of the hydantoinacrylic acid. From 5–6 hours were usually sufficient to complete the reaction and more prolonged boiling is disadvantageous. The solution in the flask, which was turbid from separation of barium carbonate but was no longer yellow, was made acid to Congo-red with sulphuric acid and filtered from barium sulphate. The solution was then concentrated to about 20 cc. on the water-bath and while still acid to Congo-red silver nitrate (5 g.) added. The small precipitate containing traces of silver chloride was filtered off and the silver salt of the amino-acid was precipitated from the filtrate as previously described for the natural acid [Dakin, 1918]. It is important to use pure alkali especially free from silica. The silver salt mixed with excess of silver oxide was filtered off and thoroughly well washed with cold water. It was then suspended in water (20 cc.) and completely decomposed with hydrogen sulphide. The filtrate from the silver sulphide was then concentrated to about 1 cc. in volume at a temperature of not over 50° . Absolute alcohol in excess was then added to the syrup with stirring and the sticky mass of amino-acid thoroughly well washed with alcohol. It was then dissolved in a few drops of cold water, in which it dissolves with the greatest ease, and reprecipitated with alcohol. The acid separates out as a white granular powder which is apt to be sticky if much water is present. It is dried *in vacuo* over phosphorus pentoxide at room temperature since it very readily passes over to hydroxypyrrolidonecarboxylic acid on heating. It was not found possible to crystallise the acid satisfactorily and its solutions when allowed to evaporate *in vacuo* gave a brittle glassy mass. The yield of amino-acid from 2 g. of hydantoinacrylic acid varied from 0.3–0.4 g.

Like the natural acid, the synthetic product gives the pyrrole reaction on heating, which is intensified on igniting with zinc dust. On coupling with diazobenzenesulphonic acid in sodium carbonate solution an orange red colour is obtained, while with sodium hydroxide as alkali, a deep cherry red is

obtained which is intensified on heating. The reaction is sensitive to about 1 : 1000. On warming with β -naphthol and excess of concentrated sulphuric acid a greenish-yellow fluorescent solution is obtained similar to that given by malic acid (Pinerua). Its conversion through loss of water on heating to 105° into a hydroxypyrrolidonecarboxylic acid is also characteristic.

As stated earlier, a small amount of the amino-acid may be obtained by the direct decomposition of hydantoin- β -bromopropionic acid with barium hydroxide, but by far the larger part of the acid undergoes profound decomposition with rupture of the carbon chain and the yield of amino-acid is extremely small. Better results may be obtained by hydrolysing the hydantoin- β -bromopropionic acid (5 g.) by heating in a sealed tube for four to five hours with five parts of fuming hydrochloric or hydrobromic acid. The contents of the tube are evaporated under reduced pressure, milk of lime is then added until the acid reaction to Congo-red is almost but not quite abolished and the diluted mixture (200 cc.) is then boiled for a couple of hours. Excess of lime (5 g.) is then added and boiling resumed until ammonia is no longer evolved. Excess of lime is removed by a current of carbon dioxide and the filtrate containing the calcium salt of the amino-acid is concentrated at low temperature to a volume of about 10 cc. The calcium salt is then precipitated by alcohol, leaving the bulk of the calcium chloride or bromide in solution. The white, very soluble calcium salt is dissolved in water, and the calcium removed exactly with sodium oxalate. Residual traces of chlorides and bromides are removed with silver nitrate after acidifying with a little sulphuric acid and then the amino-acid is precipitated as silver salt as previously described and the latter decomposed with hydrogen sulphide. The concentrated aqueous solution of the amino-acid is twice precipitated with alcohol as described in the preceding paragraph. The acid thus obtained appears to be identical with that from hydantoinacrylic acid.

In some cases, an additional precipitation of the acid was effected by precipitation of its aqueous solution with mercuric acetate in feebly acid or neutral solution. The white mercury precipitate was washed with water, then with alcohol and finally decomposed with hydrogen sulphide in the usual way. Occasionally this procedure is useful for removing traces of inorganic impurities.

For analysis, the acid was dried at room temperature over phosphorus pentoxide at 2 mm. pressure.

Analysis. 0.1979 g.; 0.2670 CO_2 ; 0.1008 H_2O ,
0.1022 g.; 0.1399 CO_2 ; 0.0483 H_2O ,
0.0100 g.; 1.46 cc. N at 17° and 752 mm. (van Slyke).

		C	H	N
Calculated for $\text{C}_5\text{H}_9\text{O}_5\text{N}$...	36.8	5.52	8.59
Found	36.8	5.66	8.34
		37.3	5.57	

On heating the acid either under normal or reduced pressure, water is evolved

freely in the neighbourhood of 100° and the substance is converted mainly into hydroxypyrrolidonecarboxylic acid. Thus on heating 46.2 mg. of the acid for one hour at $105-107^{\circ}$, the product on treatment with nitrous acid gave only 0.48 cc. nitrogen, equivalent to 0.58 % of nitrogen in the NH_2 form, indicating that about 93 % of the acid had been decomposed. An analysis of such a dried product is appended:

Analysis. 0.1168 g.; 0.1783 CO_2 ; 0.0542 H_2O .

	C	H
Calculated for $\text{C}_5\text{H}_7\text{O}_3\text{N}$...	41.3	4.9
Found	41.7	5.02

The copper salt of *i*- β -hydroxyglutamic acid is a clear blue-green salt which is very soluble in water but insoluble in alcohol. It resembles the copper salt of the natural active acid except that it appears a shade less blue and more green. It was prepared in the usual way by digesting an aqueous solution of the acid with excess of well-washed copper hydroxide. After filtering off the excess of copper oxide, the solution should be evaporated at a low temperature, as prolonged heating is apt to cause the separation of a little copper oxide. The concentrated aqueous solution of the copper salt was precipitated with alcohol and dried first over sulphuric acid and then in the oven at 100° .

Analysis. 0.1681 g.; 0.1591 CO_2 ; 0.0497 H_2O ; 0.0566 CuO ,
0.0200 g.; 2.1 cc. N at 13° and 752 mm. (van Slyke).

	C	H	N	Cu
Calculated for $\text{C}_5\text{H}_7\text{O}_5\text{NCu}$	26.7	3.12	6.23	28.3
Found	26.0	3.28	6.12	27.7

The calcium salts of *i*- β -hydroxyglutamic acid are very soluble in water but insoluble in alcohol. They are precipitated as white powders on adding alcohol to their aqueous solutions. The acid calcium salt was obtained by warming the acid with calcium carbonate in water and was found to contain 11.3 % calcium (calculated 11.0). The "neutral" salt which is strongly alkaline in reaction towards litmus, was obtained by using excess of milk of lime and precipitating with alcohol. It contained 20.0 % calcium (calculated 19.9). It is decomposed, but incompletely, by passing carbon dioxide into its aqueous solution.

The strychnine salt of *i*- β -hydroxyglutamic acid was prepared by warming a concentrated solution of the acid with twice its weight of strychnine and adding methyl alcohol (10 parts). When all the base had dissolved, the water and alcohol were removed under diminished pressure and the sticky residue was crystallised from ten parts of boiling butyl alcohol. On standing, rosettes of needles separated out, which were readily soluble in water or methyl alcohol, but less so in ethyl or butyl alcohol.

Analysis. 0.1192 g.; 0.2750 CO_2 ; 0.0666 H_2O .

	C	H
Calculated for $\text{C}_{26}\text{H}_{31}\text{O}_7\text{N}_3$...	62.8	6.24
Found	62.9	6.21

Malic semi-aldehyde.

Malic semi-aldehyde or β -hydroxy- γ -aldehydobutyric acid was obtained by the hydrolysis of its acetal, γ -diethoxy- β -hydroxybutyric acid, which was in turn obtained by the reduction of γ -diethoxyacetoacetic ester already prepared by Dudley and the writer [1914].

γ -Diethoxy- β -hydroxybutyric acid. Ethyl γ -diethoxyacetoacetate (21.8 g.) together with alcohol (20 cc.) were mixed with 180 cc. of water. The mixture was surrounded by an ice bath and thoroughly agitated with a mechanical stirrer. 4 % sodium amalgam (200 g.) was added fairly rapidly and but little gas was evolved until the reduction was complete at the end of about seven hours. The mixture was allowed to stand overnight and then saturated with carbon dioxide and evaporated almost to dryness under reduced pressure. In the earlier experiments the sodium salt of the hydroxy-acid was extracted by hot methyl alcohol in which it is freely soluble, but later this step was avoided as follows: 25 % sulphuric acid was added to the thick residue until the mixture was acid to Congo-red and an oily layer separated out. The oil was at once extracted with ether and the aqueous layer again shaken with ether. The ether extract was washed with a few drops of water, dried over sodium sulphate and evaporated. The oil which weighed about 14 g. after drying over sulphuric acid was practically pure as judged by analysis. On distilling the acid under reduced pressure, there is a good deal of decomposition; about half of it is obtained as a clear oil boiling at 120° under 5 mm. pressure.

Analysis. 0.1558 g.; 0.2860 CO₂; 0.1186 H₂O,
0.1285 g. (distilled); 0.2341 CO₂; 0.0935 H₂O.

				C	H
Calculated for C ₈ H ₁₆ O ₅	...			50.0	8.33
Found	50.1	8.45
„	49.7	8.08

The acid is a sour smelling oil suggestive of butyric and hydroxybutyric acids. It mixes readily with a little water but is precipitated on diluting, finally again dissolving in about six parts of cold water. It does not decolorise bromine even after distillation and is readily soluble in ether and chloroform, and to a noticeable extent in petroleum ether. The salts are almost all very soluble and do not crystallise easily. The *sodium salt* soluble in methyl alcohol has already been referred to. The *zinc salt* is very soluble and is not precipitated by alcohol. When its solutions are evaporated in a desiccator, it is obtained as clear brittle semi-crystalline flakes which melt at a low temperature. On adding silver nitrate to a 3 % solution of the sodium salt, no precipitation occurred in the cold but, on warming, prompt reduction took place. The acid when titrated with alkali, using phenolphthalein as indicator, gave the following result: 0.2850 g. neutralised 15.0 cc. *N*/10 sodium hydroxide, giving a molecular weight of 190 compared with a calculated value of 192.

Malic semi-aldehyde. γ -Diethoxy- β -hydroxybutyric acid (15 g.) prepared as just described was gently boiled with 50 cc. of 0.2 *N* sulphuric acid for

about an hour. After a few minutes, the whole of the oil dissolved. An amount of barium hydroxide exactly equivalent to the sulphuric acid was then added and the barium sulphate removed by filtration. The filtrate was evaporated under reduced pressure at not over 45° , when a clear syrup, readily soluble in water and alcohol but sparingly soluble in ether or chloroform, was left behind. It was impossible to distil the substance without decomposition and it did not crystallise. The yield of dry syrup was 8 g. It is inadvisable to treat the substance with strong alcohol for any length of time since either acetal or ester formation takes place easily. A portion was however dissolved in alcohol, filtered from a trace of inorganic impurities, at once diluted with water and then concentrated. It was finally dried over phosphorus pentoxide *in vacuo* and analysed with the following result:

Analysis. 0.1454 g.; 0.2185 CO_2 ; 0.0721 H_2O .

		C	H
Calculated for $\text{C}_7\text{H}_6\text{O}_4$...	40.7	5.09
Found	...	41.0	5.51

The complete purity of the product can hardly be anticipated since it could be neither distilled nor crystallised, but the results indicate no great amount of impurity. On titrating with alkali, using phenolphthalein as indicator, 0.1560 g. required 12.8 cc. *N*/10 sodium hydroxide, equivalent to a molecular weight of 122 compared with a calculated value of 118.

Malic semi-aldehyde reduces ammoniacal silver solutions on warming and instantly in the cold in the presence of sodium hydroxide. It gives a strong reaction with Schiff's reagent, especially if the reagent is dilute and not excessively acid. Bromine water oxidises the aldehyde freely, and on warming with excess of sodium hydroxide it gives inactive malic acid which was identified through its lead salt. It reacts with phenylhydrazines, but with the exception of *p*-nitrophenylhydrazine the osazones formed are not very attractive. On mixing the aldehyde in aqueous solution with semicarbazide hydrochloride, preferably without addition of alkali, a good yield of *semicarbazone* is obtained crystallising in prisms melting at 211° with effervescence.

Analysis. 0.1287 g.; 0.1630 CO_2 ; 0.0580 H_2O .

		C	H
Calculated for $\text{C}_5\text{H}_9\text{O}_4\text{N}_3$...	34.3	5.14
Found	...	34.5	5.00

Action of ammonia and hydrocyanic acid on malic semi-aldehyde. The attempts to utilise Strecker's method for the synthesis of β -hydroxyglutamic acid were made with 10 % alcoholic solutions of the aldehyde, adding anhydrous hydrocyanic acid (1 mol.) together with a few drops of alcoholic ammonia and then adding alcoholic ammonia (2 mols.) on the second day. The conditions were substantially those used by Fischer in his synthesis of serine. On the third day, fuming hydrochloric acid was added for the purpose of hydrolysing any amino-cyanohydrin that might be formed. In other experiments, the ammonia was added first and the hydrocyanic acid later, but the

results were no better. After saturating with hydrochloric acid and boiling for an hour, the mixture was evaporated to dryness and the residue extracted with alcohol, leaving most of the ammonium chloride behind. The alcohol was then removed by evaporation and the solution made alkaline with sodium carbonate and boiled to remove ammonia. The solution was then just acidified with acetic acid and precipitated with mercuric acetate. The small precipitate was decomposed with hydrogen sulphide and any β -hydroxyglutamic acid was isolated as silver salt after removing traces of chlorides. 10 g. of the aldehyde yielded about 0.25 g. of silver salt equivalent to 0.11 g. of acid and on analysis it was found to be decidedly impure. It gave, however, the qualitative reactions for β -hydroxyglutamic acid previously described, so that it is not improbable that a minute amount of the amino-acid had been formed. But it was clear that at any rate in its present form the synthesis was not a practicable one.

Experiments with derivatives of acetonedicarboxylic ester.

The direct reduction of α -isonitroso-acetonedicarboxylic ester gave unsatisfactory results, as already stated. The nitroso-compound was prepared in large quantities according to Pechmann's [1891] directions, and practically every reducing agent that had any prospect of success was tried. The reduction in either acid or neutral media gave products that on analysis furnished results similar to those required for β -hydroxyglutamic acid or its anhydride, but the product was mostly not an amino-acid as judged by the nitrous acid reaction, nor did it give an amino-acid as the main product on warming with mineral acids. For reasons already given, it was decided to attempt to lessen the reactivity of the carbonyl group of the acetonedicarboxylic ester and its nitroso derivative by condensation with orthoformic ester, since as shown in the following section, the ethoxyglutaconates thus formed may be reduced by sodium amalgam to β -hydroxyglutaric acids.

Reduction of β -ethoxyglutaconic ester. Diethyl β -ethoxyglutaconate was prepared by the condensation of acetonedicarboxylic ester and orthoformic ester with acetyl chloride as described by Claisen (cp. D.R.P. 80739) and the product was fractionated under reduced pressure. The ester (10 g.) was mixed with alcohol (75 cc.) and water (75 cc.) and then reduced with 4 % sodium amalgam (100 g.) which was added rapidly. The reaction, which was slow, was carried on at first in an ice bath with constant stirring, but later at room temperature. After standing overnight, phenolphthalein was added and the solution exactly neutralised with nitric acid. The filtered solution was then precipitated with excess of silver nitrate solution and the silver salt washed with water. A portion of the silver salt on ignition was found to contain 59.0 % of silver compared with 59.6 for silver β -hydroxyglutarate and 55.4 % for silver β -ethoxyglutarate. That β -hydroxyglutaric acid was the main or sole product of the reaction was proved by decomposing the remaining silver salt suspended in water with hydrogen sulphide, and evaporating the filtrate. The residue

crystallised completely and, after spreading on a porous plate and washing with a little ether, melted at 93–95°. The yield was 55 % of the calculated. The β -hydroxyglutaric acid was further identified by its *bis*-phenylhydrazide prepared by warming the acid with three parts of phenylhydrazine for 15 minutes at 120°. The substance crystallised from glacial acetic acid in fine white needles melting at 234–235° as described by Pechmann. The free acid was also analysed:

Analysis. 0.1265 g.; 0.1878 CO₂; 0.0623 H₂O.

		C	H
Calculated for C ₅ H ₈ O ₅	...	40.5	5.41
Found	40.5	5.47

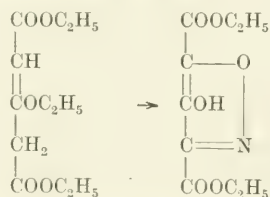
α -Nitroso- β -ethoxyglutaconic ester (?). On adding ethyl orthoformate (1 mol.) to α -nitroso-acetonedicarboxylic ester (1 mol.) no obvious reaction takes place, but on adding an acid catalyst, such as acetyl chloride or a little alcoholic hydrochloric acid, a vigorous reaction occurs with liberation of heat. On warming to 60° under diminished pressure, ethyl formate and, when acetyl chloride has been used, ethyl acetate are found in the distillate. The residual product is apparently a mixture of the α -nitroso- β -ethoxyglutaconic ester with unchanged α -nitroso-acetonedicarboxylic ester, as judged by the results of elementary analysis. The former compound requires 50.9 % carbon, 6.57 % hydrogen, the latter requires 46.7 % carbon and 5.63 % hydrogen. The observed results varied from 46.0–50.1 % carbon and 5.65–6.61 % hydrogen. It would therefore appear as if the ethoxy-compound were readily reconvertible into the ketonic ester.

The ease with which the ethyl group of ethoxyglutaconic ester could be eliminated on reduction has been shown in the foregoing. A further example is furnished by the action of excess of amyl nitrite (2 mols.) on β -ethoxyglutaconic ester (1 mol.) in the presence of 2 % of alcoholic hydrochloric acid. A crystalline product was readily separated which proved to be hydroxyisoxazolidicarboxylic ethyl ester. It crystallised from alcohol in needles melting at 104° as described by Pechmann.

Analysis. 0.1835 g.; 0.3162 CO₂; 0.0820 H₂O.

		C	H
Calculated for C ₉ H ₁₁ O ₆ N	...	47.0	4.80
Found	47.2	4.96

The reaction may be represented as follows:

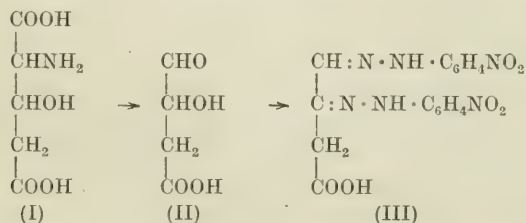


The preceding reactions clearly indicated the difficulty that was likely to be encountered in obtaining pure α -nitroso- β -ethoxyglutaconic ester starting

either from ethoxyglutaconic ester or α -nitroso-acetonedicarboxylic ester. Accordingly the products of the interaction of α -nitroso-acetonedicarboxylic ester (1 mol.) and orthoformic ester (1 mol.) after standing with a trace of alcoholic hydrochloric acid for 24 hours at room temperature were reduced in 50 % alcoholic solution without further purification, using a large excess of sodium amalgam at 0°. The reaction was kept feebly acid with sulphuric acid at first and later was allowed to become alkaline. A current of carbon dioxide was passed during the reaction which lasted 12–18 hours. As stated in the introduction, the isolation of the products of the reaction is difficult and has not been satisfactorily solved. The separation of the products by precipitating with mercuric acetate and then isolating the silver salt of the acid contained in the mercury precipitate gave a product which resembled hydroxyglutamic acid in its properties but was accompanied by other substances closely similar in elementary composition. It may be mentioned that a small amount of apparently pure β -hydroxyglutamic acid and its copper salt have been isolated with great difficulty, but much further work is needed to clear up the course of the reaction and the sharp separation of the products. The study of the reaction will be continued. The purest specimen of acid hitherto obtained by the foregoing method contained 36.4 % carbon, 5.63 % hydrogen and 8.4 % amino-nitrogen. The copper salt, which was greenish-blue resembling that obtained by alternative methods, contained 29.4 % of copper and 6.15 % nitrogen. These results are in fair agreement with the calculated figures. But this method of synthesis in its present form is much less satisfactory than that from hydantoin-propionic acid previously described.

II. THE *p*-NITROPHENYLOSAZONES OF MALIC SEMI-ALDEHYDE AND TARTRONIC SEMI-ALDEHYDE.

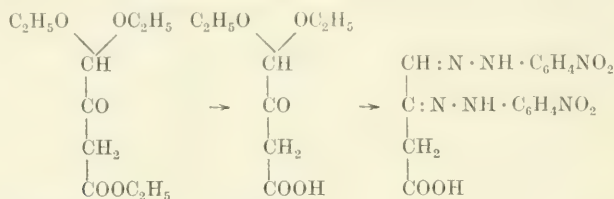
One of the main pieces of evidence which led the writer to assign to the amino-acid separated from caseinogen [1918] the structure of β -hydroxyglutamic acid was the fact that on oxidation it gave an aldehyde (II) which reacted with *p*-nitrophenylhydrazine to give a well-defined osazone (III), thus indicating that the amino and hydroxyl groups were attached to contiguous carbon atoms.



It appeared desirable, in order to confirm this deduction, to prepare the osazone in some entirely independent fashion, and this has now been done and the apparent identity of the products established. The preparation of its

lower homologue is also described, a substance which has since been obtained by the oxidation of hydroxyaspartic acid. The synthesis of the latter amino-acid from chloromalic acid will be described in a separate communication.

Malic semi-aldehyde p-nitrophenylosazone. γ -Diethoxyacetoacetic ester [Dakin and Dudley, 1914] (3 g.) was shaken with 15 cc. of normal sodium hydroxide at room temperature and the mixture allowed to stand overnight. The solution was then neutralised with dilute sulphuric acid and unchanged ester removed by extracting twice with ether. The aqueous solution was then made decidedly acid to Congo-red with more sulphuric acid and nitrophenylhydrazine (3 g.) dissolved in 15 cc. of glacial acetic acid was added. An immediate precipitate was formed and the reaction was completed by heating for 20 minutes on the water-bath. The precipitate was filtered off, washed well with warm methyl alcohol and recrystallised from boiling nitrobenzene. The yield was 1.0 g. The reactions may be represented as follows:



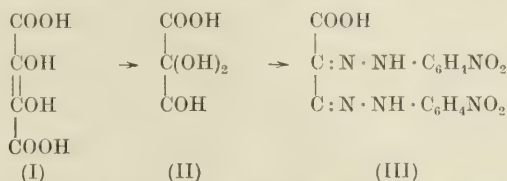
Analysis. 0.1856 g.; 0.3470 CO₂; 0.0650 H₂O.

	C	H
Calculated for C ₁₆ H ₁₄ O ₆ N ₆ ...	51.1	3.72
Found	51.0	3.89

The *osazone* is sparingly soluble in most organic solvents but it dissolves very readily in boiling nitrobenzene, crystallising out on cooling in the form of deep brown-red thick prismatic needles. It dissolves readily in pyridine even in the cold forming a salt, but is practically insoluble in water, alcohol, ether or chloroform. Its melting point varies considerably with the rate of heating and, if this is slow, may be as low as 275°, while with rapid heating it is 289–291° (uncorr.) or 297–299° (corr.). Its melting point was unchanged on mixing it with the osazone prepared from natural β -hydroxyglutamic acid as described previously [Dakin, 1918]. On adding alcoholic sodium hydroxide to a trace of the substance, a clear blue coloration is obtained, characteristic of two adjacent nitrophenylhydrazine groups.

Tartronic semi-aldehyde p-nitrophenylosazone. A solution of tartronic semi-aldehyde (II) was obtained according to Fenton's [1905] directions. Dihydroxymaleic acid (I) (1.48 g.) was added to mercuric chloride (5.5 g.) in water (15 cc.) and the mixture kept at 65–70° for an hour. The solution was then filtered from calomel and the residual mercury removed with hydrogen sulphide. Excess of hydrogen sulphide was removed by exposure to reduced pressure and then nitrophenylhydrazine (3.5 g.) dissolved in hot glacial acetic acid (15 cc.) was added. An immediate red precipitate was formed and the mixture was warmed for 20 minutes on a steam bath. The osazone (III) was

then filtered off and well washed with warm methyl alcohol. The yield was 2.2 g. The reactions may be represented as follows:



The *osazone* is sparingly soluble in alcohol, ether or chloroform, but readily dissolves in pyridine with formation of a salt which does not readily separate out. The osazone can be recrystallised from boiling nitrobenzene but it is much less soluble than the preceding osazone and the crystalline form is quite different. It separates from nitrobenzene solution in nodular rosettes of fine needles with a few separate long fine needles. The melting point of the recrystallised substance is about 302° uncorr. (310° corr.) with effervescence. Slight darkening occurs above 270°. A trace of the substance dissolved in alcoholic sodium hydroxide with an intense clear blue colour.

Analysis. 0.1780 g.; 0.3192 CO₂; 0.0570 H₂O.

		C	H
Calculated for C ₁₅ H ₁₂ O ₆ N ₆ ...		48.9	3.23
Found	48.9	3.56

An identical osazone has been obtained from the oxidation products of synthetic hydroxyaspartic acid, under the same conditions as those used for the oxidation of β -hydroxyglutamic acid.

III. OPTICAL ACTIVITY OF NATURAL β -HYDROXYGLUTAMIC ACID.

In the first communication on β -hydroxyglutamic acid [Dakin, 1918] from caseinogen it was stated that "in aqueous solution the substance shows a low dextro-rotation which is increased on addition of hydrochloric acid." The difficulties in obtaining trustworthy observations on the optical activity of the acid are mainly two-fold. In the first place, the difficulty of satisfactorily recrystallising the acid and the fact that its purification as then described rested almost as much on methods for the removal of other substances as for the direct separation of the acid in some characteristic form. The second difficulty was not appreciated at the beginning of the work and was the cause of considerable confusion until it was established that *d*- β -hydroxyglutamic acid passed over into a laevorotatory hydroxypyrrolidonecarboxylic acid on evaporating its aqueous solution or drying at 100°.

Accordingly, experiments have been made on the rotation of specimens of β -hydroxyglutamic acid, two of which were prepared from the repeatedly crystallised strychnine salt to be described in the next section, while one was obtained from the products of tryptic digestion, and all of them were carefully handled at low temperature to avoid formation of the laevorotatory anhydride. The observations are probably fairly accurate, though the possibility of the need of revision is by no means excluded.

It was found with all specimens that the free acid had a very slight dextro-rotation which was much increased in the presence of hydrochloric acid. Further, a dextro solution of the free acid on evaporation and drying at 105–110° gave a strongly laevorotatory solution which could be changed back to dextro on treating with hydrochloric acid—the first change in rotation on drying being due to the formation of hydroxypyrrolidonecarboxylic acid and the second to its reconversion into hydroxyglutamic acid. No apparent racemisation accompanied these changes. Alkaline solutions of the potassium or sodium salts of β -hydroxyglutamic acid were found to be practically inactive, but on adding Walden's uranium solution, a fairly strong laevo-rotation was observed. This behaviour shows a close analogy to malic acid and other hydroxy-acids.

Specimen A was obtained as described in a previous paper [Dakin, 1918] and was redissolved in 50 % alcohol, precipitated with mercuric acetate in feebly acid solution and the hydroxyglutamic acid obtained by decomposing the washed precipitate with hydrogen sulphide and evaporating at low temperature (25–30°). It crystallised readily and on combustion gave 37.0 % carbon and 5.57 % hydrogen. (Calculated 36.8 % carbon and 5.52 % hydrogen.)

Specimen B was obtained by the same methods as previously employed, but the caseinogen, instead of being hydrolysed with sulphuric acid, was thoroughly digested with trypsin. The yield was disappointing, only 4.5 g. being obtained from 350 g. caseinogen.

Specimens C and D were prepared similarly to A, and then converted into the strychnine salt (see next section) which was repeatedly crystallised and then decomposed with sodium carbonate. The strychnine was filtered off, and traces remaining in solution removed by shaking with amyl alcohol. The acid was then removed by precipitation with mercuric acetate in feebly acid solution, followed by decomposition with hydrogen sulphide. The polarimetric observations are recorded in the following table:

Conditions	Specimen	Concentration	Length of tube, dm.	Observed rotation	$[\alpha]_D^{20}$
Free acid in aqueous solution	A	5.43	2.2	+0.05°	+ 0.4°
" " " " " " " " " " " "	B	3.0	1.0	+0.02	+ 0.7
" " " " " " " " " " " "	C	4.0	2.0	+0.08	+ 1.0
" " " " " " " " " " " "	D	4.0	2.0	+0.10	+ 1.2
Acid in 20 % hydrochloric acid	A	2.0	2.0	+0.62	+15.5
" " " " " " " " " " " "	B	3.0	1.0	+0.48	+16.0
" " " " " " " " " " " "	C	1.33	2.0	+0.43	+16.2
" " " " " " " " " " " "	D	1.33	2.0	+0.47	+17.6
Acid dissolved in <i>N</i> potassium hydroxide	B	3.0	1.0	0	—
" " " " " " " " " " " "	C	2.0	2.0	0	—
Filtered solution from mixture containing 0.4 <i>N</i> potassium hydroxide and 12 % uranium nitrate	A	1.08	1.0	–0.13	–12.0
Ditto, 0.2 <i>N</i> potassium hydroxide, and 20 % uranium nitrate	C	0.60	2.2	–0.32	–24.3
Acid heated 2 hours 105°	A	1.73	2.0	–0.37	–10.9
" " 2 " " " " " " " " " "	B	1.0	1.0	–0.15	–15.0
" " 4 " " " " " " " " " "	C	1.20	2.0	–0.27	–11.3
* Solution C after heating with 20 % hydrochloric acid	C*	1.20	1.0	+0.20	+16.6

Taking a mean of the various observations, it may be stated that the apparent specific rotation of the free acid in 4 % solution is about $+0.8^{\circ}$, while a 2 % solution in 20 % hydrochloric acid has a rotation of $+16.3^{\circ}$. The potassium salt apparently has a negligible rotation but is strongly laevorotatory on addition of uranium nitrate. The acid becomes strongly laevorotatory on drying at 105° , but the numbers have no great quantitative value since the conversion of the hydroxyglutamic acid into its anhydride is not known to be perfectly complete.

IV. ALKALOID SALTS OF β -HYDROXYGLUTAMIC ACID AND RELATED ACIDS.

One of the main difficulties in working with hydroxyglutamic acid lay in the paucity of simple derivatives, capable of purification by repeated crystallisation. The metallic salts are either very soluble substances that require precipitation by alcohol or insoluble precipitates that are incapable of recrystallisation. It therefore became necessary to search for other salts with more acceptable properties. Of the common alkaloids only the salts of strychnine and brucine proved to be of value since the others mostly underwent hydrolytic dissociation too easily. For purposes of comparison, the corresponding salts of aspartic, glutamic and pyrrolidonecarboxylic acid have been prepared. Many of these salts are finely crystalline products and can be readily recrystallised from appropriate solvents but it is to be regretted that their melting points are not sharp in most cases, though their behaviour on heating is often characteristic.

All the salts contained equimolecular proportions of base and acid and were prepared by dissolving the acid in warm water, then adding the alkaloid till the solution became neutral to litmus, and adding a little methyl alcohol from time to time to aid in the solution of the base. The neutral solution was then evaporated at low temperature to avoid hydrolytic decomposition and recrystallised from an appropriate solvent. The specific rotation of the salts was determined in aqueous solution.

Strychnine d- β -hydroxyglutamate. The salt is very soluble in water and on slow evaporation of its solution separates out in the form of nodular masses which are spread on porous plates and recrystallised. Butyl alcohol containing a little water has been found to be decidedly the best solvent for this purpose. Methyl alcohol dissolves the salt too readily, while the solubility in ethyl alcohol is not much greater at the boiling temperature than at room temperature. 5 g. of the strychnine salt require about 60 cc. of butyl alcohol and will give about 3.5 g. of fine prismatic needles. The composition of the salt does not change on repeated crystallisation nor does its behaviour on heating. As observed in a melting point tube, a change takes place fairly sharply at a temperature varying from 165 to 175° , giving a white opaque waxy mass which does not melt till about 245° , when it gives a clear brown oil without effervescence. On dissolving the salt crystallised from butyl alcohol in water, no strychnine separates, and on evaporation long striated needles of the salt

crystallise out. These contain practically no water of crystallisation (0.3 %) and show the same behaviour on heating as that just described. The strychnine salt of β -hydroxyglutamic acid is preferable to any of the others for purposes of purification and characterisation of the acid.

Specific rotation. $C=1.67$; $l=2$ dm.; $\alpha=-0.88^\circ$; $[\alpha]_D^{20}=-26.3^\circ$.

Analysis. 0.1629 g.; 0.3760 CO₂; 0.0963 H₂O,
0.1145 g.*; 0.2640 CO₂; 0.0630 H₂O.

* Recrystallised five times.

	C	H
Calculated for C ₂₆ H ₃₁ O ₇ N ₃	62.8	6.24
Found	62.9	6.57
"	62.9	6.11

Brucine d- β -hydroxyglutamate. This salt separates out in the form of needles containing water of crystallisation on evaporation of its aqueous solution at low temperatures. It is best purified by spreading on a porous tile, then warming with acetone in which it is insoluble and recrystallising the residue either from a little water or from methyl alcohol. The salt crystallised from water begins to soften at about 90° and melts to a turbid fluid around 110°. The anhydrous salt obtained as prismatic needles by crystallisation from dry methyl alcohol decomposes indefinitely above 200°. Both salts are extremely soluble in water and alcohol but almost insoluble in acetone.

Specific rotation. $C=1.0$; $l=2.0$; $\alpha=-0.50^\circ$; $[\alpha]_D^{20}=-25.0^\circ$.

Analysis. 0.1540 g.; 0.3368 CO₂; 0.0905 H₂O.

	C	H
Calculated for C ₂₈ H ₃₅ O ₉ N ₃	60.3	6.26
Found	59.6	6.53

Strychnine d-glutamate. The salt is prepared as usual but the neutral solution should be concentrated at low temperatures and methyl alcohol added by degrees to replace the water. A syrupy mass results which, on mixing with excess of absolute alcohol, gives stout isolated glistening prisms which dissolve in water without separation of strychnine. The salt is practically insoluble in dry methyl, ethyl or butyl alcohols, but extremely soluble in water. It begins to show signs of decomposition on heating above 200° and melts about 225–230°.

Specific rotation. $C=2.0$; $l=2.0$; $\alpha=-1.02^\circ$; $[\alpha]_D^{20}=-25.5^\circ$.

Analysis. 0.2217 g. dried at 100°; 0.0196 N (Kjeldahl)=8.84 % N.

Calculated for C₂₆H₃₁O₆N₃ 8.69 % N.

Brucine d-glutamate. The salt crystallises from water in the form of long stout prismatic needles with a satin lustre. These crystals contain about 15.5 % of water of crystallisation which is lost at 100°. This corresponds to between five (14.2 %) and six molecules (16.4 %) of water. The salt as crystallised from water dissolves readily in hot methyl alcohol and, on cooling, bright rhombic prisms of the anhydrous salt separate out. The water-containing salt dissolves less readily in ethyl or butyl alcohol. After recrystallisation from

methyl alcohol, the anhydrous salt is soluble only with great difficulty in any of the alcohols. The salt containing 5.5 mols. of water softens at about 96° and melts at about 101°, while the anhydrous salt slowly decomposes above 170° but does not melt until about 240°.

Specific rotation. $C=2.0$ (anhydrous); $l=2.0$; $\alpha = -0.92^\circ$; $[\alpha]_D^{20} = -23.0^\circ$.

Analysis. 0.2 g. dried at 100°; 0.0154 N (Kjeldahl) = 7.70 % N.
Calculated for $C_{23}H_{33}O_8N_3$ 7.76 „

Strychnine l-aspartate. The salt is very soluble in water and separates in crusts which have the appearance of stout prisms to the naked eye but are made up of masses of fine interlacing needles. Its solubilities closely parallel those of the corresponding glutamate and it melts fairly sharply to a brown oil with effervescence around 252–255°.

Specific rotation. $C=1.164$; $l=2.0$; $\alpha = -0.66^\circ$; $[\alpha]_D^{20} = -28.3^\circ$.

Analysis. 0.1500 g.; 0.0134 N (Kjeldahl) = 8.90 % N.
Calculated for $C_{25}H_{29}O_6N_3$ 9.0 „

Brucine l-aspartate. As first obtained from aqueous solution, this salt forms rather waxy prisms which become hard and brittle on drying and take on an opaque white appearance. It contains 5 molecules of water of crystallisation which are given off at 90°. (Water: found 14.9 %, calculated 14.6.) The salt is readily soluble in methyl alcohol but does not crystallise out readily on cooling. On adding ethyl acetate, in which it is sparingly soluble, it is deposited as fine needles. The anhydrous salt is moderately soluble in ethyl alcohol and less so in butyl alcohol, but does not crystallise out readily. It is insoluble in acetone. The salt containing water of crystallisation softens at 96° and melts about 100°, while the anhydrous salt decomposes indefinitely above 200°.

Specific rotation. $C=1.702$ (anhydrous); $l=2.0$; $\alpha = -0.97^\circ$; $[\alpha]_D^{20} = -28.4^\circ$.

Analysis. 0.1500 g.; 0.0121 N (Kjeldahl) = 8.05 % N.
Calculated for $C_{27}H_{33}O_8N_3$ 7.97 „

Strychnine l- α -pyrrolidone-carboxylate. The acid used for preparing this and the succeeding salt was obtained by heating glutamic acid under reduced pressure at 165° and then crystallising the products from alcohol. It had a specific rotation in water of -5.7° , and hence was partially racemised since the pure laevo-acid is generally regarded as possessing a rotation of about -7.2° .

The salt crystallises from water in the form of felted fan-shaped masses of fine needles which are readily soluble in ethyl alcohol but sparingly soluble in acetone. It dissolves easily in butyl alcohol and also in chloroform but crystallises better from water than from any of the organic solvents. The salt thus obtained softens on heating at 90° and partially melts. It contains water of crystallisation which is lost at 100°. The anhydrous salt melts indefinitely but incompletely until about 245° is reached.

Specific rotation; $C=2.74$; $l=2.0$; $\alpha = -1.45^\circ$; $[\alpha]_D^{20} = -26.7^\circ$.

Analysis. 0.2438 g.; 0.0218 N (Kjeldahl) = 8.96 % N.
Calculated for $C_{26}H_{29}O_5N_3$ 9.07 „

Brucine 1- α -pyrrolidone-carboxylate. This salt is better suited than the preceding one for purposes of characterisation of the acid. On concentrating the neutral aqueous solution, a syrup was obtained which at once solidified to a mass of stout prisms. The salt is very soluble in methyl alcohol but can be conveniently crystallised from boiling acetone from which it separates in well-formed prisms. On heating the substance, decomposition can be noted as low as 140° and it melts with effervescence to a clear fluid from 180–195°.

Specific rotation. $C=5.0$; $l=2.0$; $\alpha=-3.15^\circ$; $[\alpha]_D^{20}=-31.5^\circ$.

Analysis. 0.4619 g.; 0.0364 N (Kjeldahl) = 7.88 % N.

Calculated for $C_{28}H_{33}O_7N_3$ 8.03 ..

V. IDENTIFICATION OF β -HYDROXYGLUTAMIC ACID AMONG THE PRODUCTS OF HYDROLYSIS OF GLUTENIN AND GLIADIN.

The experiments in the following section were made with the cooperation of Miss Marjorie Lord Strauss, to whom acknowledgment is due for much valuable assistance.

Hydroxyglutamic acid up to the present has only been isolated from caseinogen, a typical animal protein, after hydrolysis with acids, with trypsin, and possibly to a smaller extent with barium hydroxide. There seems every reason to suppose that search for this amino-acid will disclose its presence in other proteins. The present communication records its presence in two typical vegetable proteins, glutenin and gliadin.

The protein preparations were prepared from wheat flour according to the excellent methods described by Osborne. The methods employed were practically identical with those described in the work on caseinogen [Dakin, 1918]. Portions of 100 g. of each protein were hydrolysed by prolonged boiling with dilute sulphuric acid and the latter subsequently removed exactly with barium hydroxide. The concentrated solution of amino-acids was then thoroughly extracted with butyl alcohol and the aqueous residue containing essentially the strong acids and bases was saturated with hydrochloric acid to separate the bulk of the glutamic acid. The calcium salts of the acids were then separated by alcohol according to Foreman's method. They were dissolved in water, the calcium removed exactly with oxalic acid, and the diluted filtrate boiled with lead hydroxide to remove aspartic acid. Lead was removed from the filtrate with hydrogen sulphide and traces of bases precipitated with phosphotungstic acid in sulphuric acid solution. After removal of the excess of the latter reagents with barium hydroxide, the filtrate was made acid to Congo-red with sulphuric acid and concentrated to about 100 cc. The silver salt of β -hydroxyglutamic acid was then separated in the usual way and decomposed with hydrogen sulphide. The crude acid, as thus separated, was less pure than when prepared from caseinogen. It was purified by dissolving in cold 50 % methyl alcohol and precipitating with mercuric acetate in very feebly acid (acetic) solution. The precipitate, on decomposition with hydrogen sulphide and concentration at not over 50°, gave a very soluble syrup which

solidified in the desiccator and had all the properties of β -hydroxyglutamic acid prepared from caseinogen. The yield of acid was small but undoubtedly the losses were considerable. 100 g. of gliadin gave 2.4 g. of the acid, while 1.8 g. were obtained from 100 g. of glutenin. It is worth noting that the yields of the acid from caseinogen have been found to vary enormously. At times, not more than 2-3 % has been obtained in a reasonably pure state, while in other experiments as much as 10 % has been obtained. It is not unlikely that the acid, being a β -hydroxy-acid is not entirely stable to many of the reagents employed. The yields are generally highest when small amounts of protein are worked up at a time.

Acid from gliadin.

Analysis. 0.2112 g.; 0.2861 CO₂; 0.1100 H₂O.

	C	H
Calculated for C ₅ H ₉ O ₅ N...	36.8	5.52
Found	36.9	5.79

On heating the acid previously dried over phosphorus pentoxide to 110-115°, water was split off and the residue, containing 40.9 % of carbon and 5.3 % of hydrogen, was mainly made up of hydroxypyrrolidonecarboxylic acid, for it contained less than 1 % of nitrogen in the (NH₂) form as judged by van Slyke's method with nitrous acid.

The optical properties of the acid resembled those previously recorded. In aqueous solution $[\alpha]_D^{20} = +1.0^\circ$ ($c = 5.87$, $l = 2.0$, $\alpha = +0.11^\circ$). In 20 % hydrochloric acid ($c = 1.96$), $[\alpha]_D^{20} = +14.0^\circ$.

Acid from glutenin. The acid obtained from glutenin resembled the preceding closely. It contained 37.0 % carbon and 5.8 % hydrogen. It showed the characteristic decomposition on heating at 110° but its concentrated aqueous solution showed no detectable rotation in 10 % aqueous solution and in 20 % hydrochloric acid it had $[\alpha]_D^{20} = +6.2^\circ$. It appeared therefore as if partial racemisation had taken place in the process of preparation, for careful investigation failed to indicate the presence of any other acid. The strychnine and brucine salts were also prepared and analysed without disclosing any other cause for the lower rotations. It is not unlikely that racemisation may have been caused by the alkali used in the preparation of the glutenin.

VI. THE FATE OF β -HYDROXYGLUTAMIC ACID IN THE DIABETIC ORGANISM.

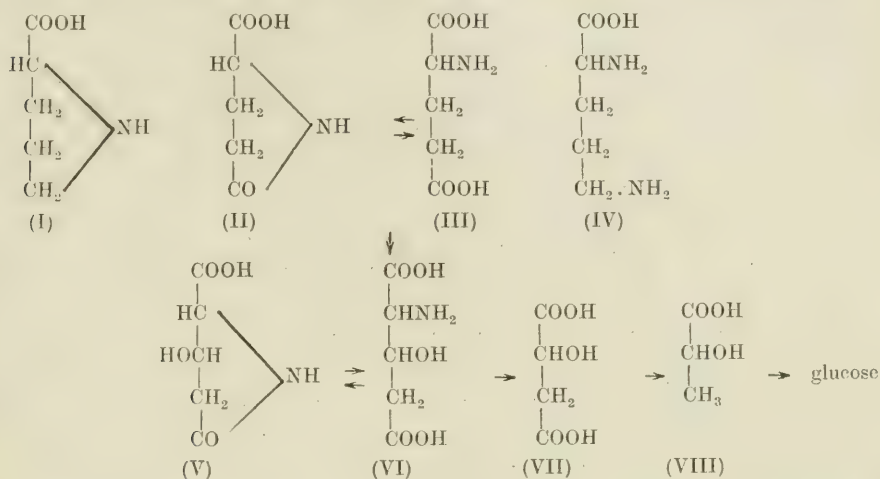
The fate of β -hydroxyglutamic acid in the diabetic animal presents certain questions of interest. Lusk has shown that when glutamic acid (III) is fed to a starving dog fully under the influence of phloridzin, about 65 % of the weight of glutamic acid given reappears in the urine as "extra glucose." The writer has shown that substantially the same thing is true for proline (I) and ornithine (IV), both of which, like glutamic acid, contain five carbon atoms. It appears as if three out of the five carbon atoms were concerned in glucose formation, and it is not unreasonable to regard it as likely that their catabolic paths are similar.

Now β -hydroxyglutamic acid has been found to yield glucose in the diabetic animal in precisely the same fashion as the previously mentioned acids. This fact appears to the writer to possess a certain speculative significance based on the probability of similarity in the metabolism of all of these acids. In the first place, if, as appears likely, β -hydroxyglutamic acid may arise in the body from glutamic acid, an example is furnished of " β -oxidation" such as is known to occur in the oxidation of fatty acids, but has not hitherto been observed with the amino-acids. Secondly, the possible conversion of β -hydroxyglutamic acid into malic acid (VII) by oxidation must be regarded as plausible for it involves a type of simple reaction often encountered in animal oxidation, and has, moreover, been effected by the writer *in vitro*. Malic acid is also stated to occur in sheep's sweat in significant quantities. Now Ringer, Frankel and Jonas [1913] have shown that malic acid readily goes over into glucose in the diabetic dog, 13 g. of the acid furnishing 8 g. of glucose. A similar conversion of malic acid into sugar has been observed in acorns by Bloor [1912]. It is therefore not unlikely that malic acid is a product of the intermediary metabolism of hydroxyglutamic acid and, in turn, of the other 5-carbon acids. In picturing the possible further steps in the conversion of malic acid into glucose, one cannot avoid considering favourably the idea that it may part with carbon dioxide with formation of lactic acid (VIII), for this is a reaction which is actually known to occur as the result of enzyme activity in the case of vegetable cells. This change is also observed in the ageing of wines and is apparently affected by *Micrococcus malolacticus* [cp. von der Heide, 1912]. The subsequent conversion of lactic acid into glucose involves no difficulty.

One other point may be referred to in connection with the rôle of β -hydroxyglutamic acid in metabolism. The conversion of proline into glucose obviously requires the opening of the pyrrole nucleus. The reversibility of many biological reactions lends significance to the extraordinarily easy conversion of β -hydroxyglutamic acid into hydroxypyrrolidonecarboxylic acid—and the less easy conversion of glutamic acid into α -pyrrolidonecarboxylic acid (II). Such changes may well be concerned with the synthesis of substances containing the pyrrole nucleus, and, moreover, the reduction of pyrrolidonecarboxylic acids to pyrrolidinecarboxylic acids is known to be feasible *in vitro* from Fischer's experiments.

It should be noted that Ringer, Frankel and Jonas have described experiments which they consider prove that glutamic acid is converted into succinic acid in the body, while they regard malic acid as an intermediate product in the metabolism of aspartic acid. They picture the malic acid giving hydroacrylic acid and malonic acid, but curiously enough ignore the possibility of its giving lactic acid in spite of the fact that malonic acid gives little or no glucose in the diabetic animal. The writer is completely unable to follow the logic of Ringer's structural explanations of glucogenesis and cannot help feeling that too little distinction is made between possible metabolic paths and those actually known to occur.

The relationships in structure of the various five-carbon acids and their possible conversion into glucose *via* malic and lactic acids as previously outlined may be seen from the following scheme:



Experimental. Two experiments were made on the administration of β -hydroxyglutamic acid to a diabetic dog. The conditions of the experiments were those used previously by the writer [1913]. Phloridzin was administered to the starving dog by Coolen's method. 1 g. emulsified in 10 cc. of olive oil was given every day. The urine was collected in 18 hour periods and the bladder was carefully catheterised and washed out with sterile salt solution at the end of each period. The acid was given as the mono-sodium salt in aqueous solution by subcutaneous injection. The glucose was determined gravimetrically and checked by polarimetric readings. The excretion of "acetone bodies" was followed but showed no special change, hence the results are not recorded. The animal used was a terrier dog weighing about 8 kilograms.

Period	Hours	Total N	Glucose	G/N ratio	Substance
I	—	—	—	2.80	
II	18	4.21	13.53	3.22	
III	18	5.32	21.90	4.11	17.4 g. acid = 1.5 g. N
IV	18	5.10	15.74	3.08	
V	18	5.47	19.79	3.62	10 g. acid = 0.86 g. N

The "extra glucose" following the administration of the β -hydroxyglutamic acid may be calculated approximately using Lusk's method. The $\frac{\text{Glucose}}{\text{Nitrogen}}$ ratio in the blank periods averages 3.1. Assuming that the whole of the nitrogen of the amino-acid given is excreted in the course of the following 18 hour period, it is calculated that in the first experiment 17.4 g. of the acid gave rise to 10.06 g. of "extra glucose" while 10 g. of the acid gave 5.5 g. From the mean of the experiments it is concluded that β -hydroxyglutamic acid may furnish about 55–60 % of its weight of glucose in the diabetic dog.

According to the suggestion advanced that β -hydroxyglutamic acid may yield glucose in the diabetic animal *via* lactic acid, it may be calculated that the theoretical yield is 55 %, assuming one molecule of glucose to arise from two of lactic acid and two of hydroxyglutamic acid. The almost precise coincidence of the observed and calculated result must be regarded as partly accidental since the unavoidable experimental errors are considerable, but even allowing for them, the agreement is striking. The "protein sparing" effect of the injected amino-acid is very obvious.

SUMMARY.

Considerable difficulty has been experienced in synthesising inactive β -hydroxyglutamic acid.

Bromination of the acetyl derivative of β -hydroxyglutaric anhydride and subsequent action of ammonia on the product did not yield the desired result.

Small amounts of the amino-acid were obtained on reducing α -isonitroso-acetonedicarboxylic ester but the results were unsatisfactory.

Only traces of the amino-acid were formed by the application of Strecker's reaction to malic semi-aldehyde.

The most satisfactory synthesis so far accomplished had as its starting point glutamic acid. The steps are as follows: glutamic acid \rightarrow α -uraminoglutamic acid \rightarrow hydantoin-propionic acid \rightarrow hydantoin- β -bromopropionic acid \rightarrow hydantoinacrylic acid \rightarrow β -hydroxyglutamic acid.

It is noted that hydroxyaspartic acid has been synthesised from barium chloromaleate.

The hitherto unknown semi-aldehyde of malic acid has been prepared from γ -diethoxyacetoacetic ester. Its *p*-nitrophenylosazone and that of tartronic semi-aldehyde are described.

The optical properties of β -hydroxyglutamic acid have been examined. The specific rotation of the free acid in 4 % aqueous solution is about $+0.8^\circ$, while a 2 % solution in 20 % HCl has a specific rotation of $+16.3^\circ$. The rotation of the potassium salt is practically undetectable, but the addition of uranium nitrate renders the solution strongly laevorotatory. On drying the free acid at 105° it loses water and is converted into hydroxypyrrolidonecarboxylic acid, which has a strong laevo-rotation in aqueous solution.

The strychnine and brucine salts of the following acids are described: *d*- β -hydroxyglutamic, *d*-glutamic, *l*-aspartic, and *l*- α -pyrrolidonecarboxylic.

β -Hydroxyglutamic acid has been isolated from gliadin and glutenin.

When administered to a diabetic (phloridzinised) dog β -hydroxyglutamic acid yields 55–60 % of its weight as glucose, apparently three of its five carbon atoms being concerned in glucose formation. In this it resembles glutamic acid, proline and ornithine, and it seems not unreasonable to regard the catabolic paths of all these acids as similar. The possibility of their conversion into glucose *via* malic and lactic acids is indicated.

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XXXVIII. STUDIES IN THE ACETONE CONCENTRATION IN BLOOD, URINE, AND ALVEOLAR AIR: I. A MICROMETHOD FOR THE ESTIMATION OF ACETONE IN BLOOD, BASED ON THE IODOFORM METHOD.

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SCANDINAVIAN readers will find in my book [Widmark, 1917] a summary of the most important methods hitherto used for the estimation of the acetone concentration in blood and tissues. It will be found that none of the methods there referred to is entirely satisfactory or applicable to more extended measurements. Series of estimations upon living material cannot be obtained.

Of a method that is to be used both in the physiological laboratory and in clinical work the following demands, among others, should be made:

(a) It should not require more than about 100 mg. of blood. (b) The carrying out of the estimation should be simple, and the apparatus not too complicated or expensive. (c) The error should not exceed 10 %.

In the blood of diabetics with fairly pronounced acidosis the concentration of the total acetone fluctuates among values lying at about 0.20 per mille, *i.e.* in 100 mg. of blood there are about $20\gamma^1$ total acetone. A micromethod should therefore have a mean error in the single estimation not greatly exceeding about $\pm 2\gamma$, which would therefore constitute an error of 10 % with a concentration of 0.20 per mille.

A detailed investigation of the iodoform method has shown that a modification of this renders possible an estimation of these small quantities of acetone with a mean error which remains approximately that just named. The carrying out of the estimation is simple and requires little practice. No more than 100 cmm. of blood is required, and the apparatus is not complicated.

We shall now describe the process, giving first the main outlines of the method and the carrying out of the estimation, and in a further section entering into a detailed examination of the method.

¹ We use the symbol γ for the quantity 0.001 mg., according to the proposal of the "Ausschuss für Einheiten und Formelgrößen" [1913].

A. DESCRIPTION OF THE MICROMETHOD.

1. *The method of obtaining the blood sample.*

The requisite amount of blood is obtained in the usual way by pricking with a stilette the ball of the finger or the ear-lobe. In disinfecting the skin care should be taken to remove all traces of alcohol and ether before the puncture is made. It is best merely to wash with soap and water. With a capillary pipette, graduated up to 100 cmm., the blood is sucked up as it issues from the wound. It is of great importance that the blood should not be subjected to evaporation, since in that case too low values may be obtained. The sucking up and measuring of the blood may be carried out most easily and most quickly by the aid of a precision regulator as constructed by Weichardt [1909]¹. The blood is brought into the flask of the distillation apparatus, in which there has previously been placed 10 cc. of 1 % phosphoric acid. The pipette is rinsed out four or five times with the phosphoric acid, so that every trace of blood passes down into the flask, which is immediately corked up.

The pipette should not be cleaned with alcohol and ether. The best method is to place it in the tube of a vacuum pump. After distilled water has been sucked through the pipette for a few moments, wipe the point. After a few minutes' further suction the pipette will be dry.

2. *The distillation.*

The distillation should be undertaken not more than two or three hours after the sample of blood has been secured. The shape of the distillation apparatus is shown by Fig. 1. From the distillation flask, the capacity of which is 100 cc., passes a detachable, twice-bent distillation tube. The tube is provided with a small drop-receiver and is blown out, immediately under the second angle, to a bulb of a few cc. capacity. It is drawn out at the end into a point the opening of which is about $\frac{1}{4}$ mm. across. A test-tube 150 mm. long and 15 mm. wide serves as receiver. The test-tube is enclosed by a cooler, the form of which is shown by the figure. The distillation tube reaches down into the test-tube and should end a couple of mm. above the bottom. The flask, distillation tube, and test-tube with accompanying cooler can be fixed in a wooden stand with elastic metal clamps in such a way that the test-tube can be loosened and withdrawn from the stand by a single movement.

In the test-tube, immediately before distillation, are placed 3 cc. of *N*/2 sodium hydroxide solution [natr. hydricum puriss. e natrio], and 2 cc. of *N*/200 iodine solution are added from a well-calibrated pipette (or in the estimation of greater quantities of acetone than 50γ, 2 cc. of *N*/100 iodine solution).

¹ A modification of this is sold by the A. B. Vetenskapliga Instrument (Scientific Instrument Company), Lund, Sweden.

Before distillation, the flask and the distillation tube should be boiled out for a few minutes with 1 % phosphoric acid. This is especially important if a cork connection is used between the tube and the flask.

In the distillation of pure water or phosphoric acid solution the strength of the titer of the alkaline iodine solution is diminished to some extent. In order to determine the titer two blind tests are carried out before the distilling

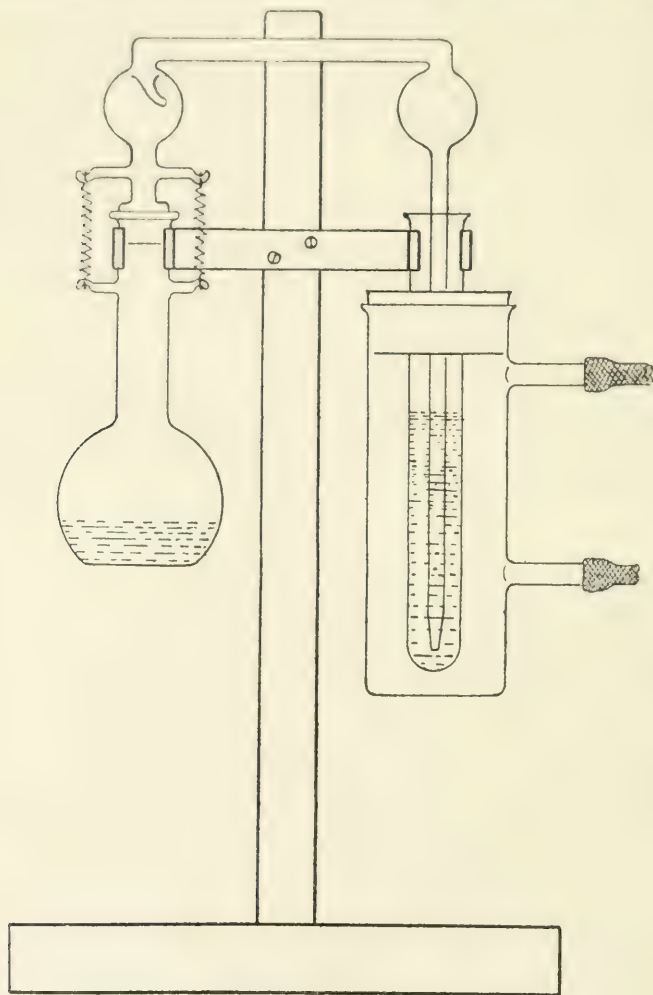


Fig. 1

of the blood sample, and one blind test afterwards. In these 10 cc. of 1 % phosphoric acid are placed in the flask, and the distillation is carried out as described below for the blood sample. The first blind test usually shows too great a consumption of iodine and should not be counted in the determination of the titer. The last will serve as a test by which it may be ascertained if any error has slipped in during the distillation of the blood sample.

The distillation of the blood sample is carried out as follows. The cork is removed from the flask and the distillation tube is attached. The apparatus is placed in the stand, and the condensing water turned on. The fluid is then boiled, the burner being held in the hand and the flame kept small. As soon as the steam has reached the contents of the test-tube a loud rattling will be heard. From this moment the boiling is continued for 100 seconds, the heat being moderated by raising and lowering the burner, so that the boiling remains even and the blood does not froth up into the distillation tube. A test of the evenness of the boiling exists in the rattling noise, which ought not to cease. When the 100 seconds are up, the heat is increased a little and at the same time the test-tube is loosened from its clamp, so that the point of the distillation tube comes above the surface of the fluid. No fluid must be allowed to remain clinging to the point; any that does so remain can be blown down into the test-tube by heating the flask for a moment. When the test-tube has been further cooled down for a few minutes, the condensing water is shut off and the test-tube is taken out of the cooler. We may now proceed to the next distillation; 10 distillations can be carried out in an hour. A precipitate appears only on the distillation of quantities of acetone exceeding 50γ , the iodoform being to a certain extent soluble in water.

3. *The titration.*

When the tube has stood for at least three minutes, 3.5 cc. of $N/2$ sulphuric acid are added from a burette. In every tube is placed a thin glass rod, the lower end of which is flattened out into the form of a disc, in order to facilitate the stirring of the fluid. The solution is stirred by moving this glass rod a few times up and down. A couple of drops of a 1 % starch solution are now added with a pipette, and titration is carried out with $N/200$ sodium thiosulphate solution (or $N/100$ if $N/100$ iodine solution be used). In the titration we use a burette (Bang's burette for the estimation of blood sugar) graduated in 0.02 cc. Thiosulphate is added drop by drop as the fluid is stirred with the glass rod, until the blue colour almost vanishes. Towards the end of the titration only fractions of a drop are added. The titration is continued until every trace of colour has vanished; the easiest way to find out when this is the case is to look at the fluid through the mouth of the test-tube. With a little practice the change can be determined to somewhat less than half a drop. Care must be taken that no drops of thiosulphate remain clinging to the upper parts of the test-tube. They can be brought down with the glass rod.

The blind test enables us to determine the titer of the iodine solution. The amount of acetone is calculated from the difference between the quantities of thiosulphate used in the blind test and in the blood test. 0.02 cc. of $N/200$ thiosulphate corresponds to 0.968γ acetone.

B. DETAILED EXAMINATION OF THE METHOD.

For the testing of the method pure acetone was used, prepared in the following way from the bisulphite compound [see Plimmer, 1915]. To 125 cc. of crude acetone were added 70 g. of sodium bisulphite in saturated solution. The mixture was well shaken in a closed flask and, after being left to stand for some time, the liquid was drawn off by means of a suction filter and the precipitate dried between filter-paper. It was then placed in a distillation flask and decomposed with 40 g. of soda. The mixture was subjected to fractional distillation, and the distillate was dried with calcium chloride and subjected to a fresh distillation. The fraction that passed over at a temperature of between 56 and 57° was used in the experiments.

The pure acetone boiled at a temperature of + 56·8°, and had a sp. g. of 0·798 at 15°. Its reaction was neutral and it showed no aldehyde reaction (equal volumes of 10 % silver nitrate and sodium hydroxide were mixed together, ammonia was added drop by drop until the precipitate was dissolved, and a couple of drops of acetone were then added. No mirror was formed).

A solution prepared from 1·462 g. acetone in 1 litre of water showed the following concentration, when titrated by Messinger's method:

1·465 ± 0·0023 per mille (6 estimations).

The acetone may therefore be regarded as sufficiently pure for the testing of the micromethod.

In order to render possible the preparation of an acetone solution of accurately known concentration and to avoid any loss through evaporation in the measuring of the pure acetone, the following method of procedure was adopted.

A number of small bulbs of 1–2 cc. capacity were blown out from narrow glass tubes and the point of each bulb was drawn out into a capillary tube. The bulb, after being weighed, was filled with the pure acetone by gently heating it and then dipping the capillary into the acetone. As cooling took place the bulb was filled with the fluid. The capillary was then melted off, and the filled bulb weighed again.

In the preparation of dilute acetone solutions a 1 litre volumetric flask was half filled with water, and a bulb was dropped into the water and broken with a glass rod. The flask was then filled up to the mark. Finally there was further added a small quantity of water corresponding to the volume of the glass of the bulb, the sp. g. of which was assumed to be 2·5.

1. *Measurement of the blood sample.*

The greatest risk of error in the measurement of the blood sample lies in the fact that the acetone easily volatilises. Opportunity for this is found in the time which elapses from the moment when the blood comes out of the puncture until it is sucked up into the pipette. In the pipette the danger of evaporation is extremely small.

Generally I have proceeded in the following way: 4-5 drops of blood from the puncture were allowed to drop on to a clean watch-glass, from which the blood was quickly sucked up into the pipette. The time required from the falling of the first drop of blood upon the glass until the requisite quantity had been sucked up into the pipette usually ran to 15-20 seconds. During this period the evaporation is so small that it is not noticeable with the micromethod.

This method has however been sharply criticised, and it must be granted that the method is associated with a certain risk for persons without practice. I recommend therefore that the blood be not allowed to drop upon a watch-glass. Instead it may be sucked up directly by placing the point of the pipette over the puncture and sucking up the blood as it drips out. This can be done very easily and conveniently by means of the Weichardt regulator mentioned above. With a blood sample from the ear of the rabbit it is best to let an assistant hold the animal's ear stretched out upon a square of glass. This will give the necessary support and firmness for the sucking up of the blood as it drips out.

That the taking of the sample in this way gives a perfectly satisfactory result is shown by the following experiments.

Experiment 1. A quantity of acetone was subcutaneously injected into a rabbit. Three hours later seven blood samples were taken; nos. 1, 3, 5 and 7 by means of the pipette, nos. 2, 4 and 6 by direct dropping of the blood from the ear vein into weighed flasks. Room temp. 17°.

The samples taken with the pipette gave the following values:

1	0.392 ‰
3	0.392
5	0.390
7	0.387
Average	0.390

The weighed samples gave the following results:

2	123.2 mg. blood contained	46.5γ acetone	=0.377 ‰
4	66.6	24.4γ	=0.366
6	124.0	44.0γ	=0.355
	Average		0.366

Experiment 2. Similar experiment with a greater quantity of acetone. Room temp. 16.5°.

With the pipette:

2	0.629 ‰
4	0.624
6	0.615
8	0.620
Average	0.622

By dropping directly into weighed flasks:

1	68.8 mg. blood contained	43.3γ acetone	=0.629 ‰
3	84.4	50.3γ	=0.596
5	113.4	67.8γ	=0.598
7	106.0	67.3γ	=0.635
9	80.2	46.9γ	=0.585
	Average		0.609

By weighing, somewhat lower values are found than when the pipette is used, this being evidently due to the fact that in the former case the blood is measured in mg., in the latter in cmm. If we assume the sp. g. of the blood to be 1.05 and calculate the weighed samples according to this, we find the average in Exp. 1 to be 0.384 ‰ and in Exp. 2 0.639 ‰, the former value a little lower, the latter a little higher, than the average of the samples measured with the pipette.

The measurement with the pipette is simpler and easier than weighing and is therefore to be preferred.

The risk of evaporation however is sufficiently great to put out of the question a sucking up of the blood and weighing with a torsion balance in the same way as in the blood-sugar test according to Bang. On the other hand it is possible that this method may be very well suited to an estimation of aceto-acetic acid. For this purpose the blood may be sucked up on to thin filter-paper and weighed, and the free acetone may be allowed to evaporate either by letting the filter-paper hang for some time under ordinary atmospheric pressure, or by enclosing it for a few minutes in a flask which is then evacuated by means of a water suction pump. This process is at present being worked out, but the details are not ready for publication. If Shaffer's [1911] method for converting β -hydroxybutyric acid into acetone and then estimating the acetone should prove to be practicable, this micromethod could probably also be used with advantage for a micro-estimation of β -hydroxybutyric acid in the blood.

2. *The distillation.*

The distillation of the blood sample presents no noteworthy difficulties. The construction of the distillation apparatus may vary in different ways. An indispensable condition however is that the steam must not be allowed to come into contact with either rubber stoppers or tubes. It seems, in fact, as if the steam possessed the power of carrying along with it substances which unite with iodine. The consequence of this is that the estimations become too high: the error may often amount to several tenths per cent of the real value. Cork may be used, but it must be well boiled previously. It is safest to use an apparatus entirely of glass, with distillation tube ground in. The disadvantage of such an apparatus however is that it is impossible without great expense to get a considerable number of distillation flasks of the right size to fit into one and the same distillation tube. This fact makes it difficult to undertake series of estimations.

The advantage of the apparatus previously described is that it is particularly simple both in construction and management. I have generally used cork connections between distillation tube and flask. This was the case in all the estimations in this part of the work. Care should be taken that the open end of the distillation tube is not too large, and that the tube does not end more than 4 mm. above the bottom of the test-tube.

The fact that I allow the acetone distilled over to be absorbed without previous cooling by the alkaline iodine solution, which in the course of the distillation has been heated to a considerable extent, might possibly lead to the supposition that great loss would be unavoidable, or at least would very easily arise. Practical experience however shows—as appears quite clearly in the chapter on the precision of the method—that the risk of a loss of acetone is very small. Moreover

there is also some theoretical support for the view that the absorption of the acetone ought not to be impaired by a gentle heating of the alkaline iodine solution. The decisive tests for the retention of the acetone in the iodine solution are in the first place its vapour-pressure in the solution, and secondly the velocity of the reaction in the formation of iodoform. With a rise of temperature both these factors are intensified, but they act in opposite directions. The increased vapour-pressure facilitates the evaporation of the acetone, the increased speed of the reaction renders it possible for greater quantities of acetone to be bound as iodoform within the unit of time. The loss of acetone will depend on the question which of these two factors is increased most with a given rise of temperature. According to Regnault [1862] the tension of the acetone is increased from 179.6 mm. Hg at 20° to 420.2 mm. at 40°, a rise therefore in the proportion of approximately 1 : 2. The partial pressure in an aqueous solution of 1 % is increased, according to my own measurements, in approximately the same proportion with a rise in temperature from 20 to 40°. If we assume that the velocity of the reaction in the formation of iodoform follows van't Hoff's law, the velocity for a rise in temperature of 20° should be increased in the proportion of 1 : 4. Hence it follows that the retention of the acetone in the alkaline iodine solution is facilitated to an appreciable extent by heating. In this connection however another circumstance of great importance is to be mentioned. It is an established fact that when iodine is added to alkali the resulting solution does not remain unchanged if left to stand. The hypiodite formed at the beginning passes over by degrees into iodate:



The iodate does not form iodoform with acetone. The formation of iodate proceeds slowly in cold solutions, but almost instantaneously in warm. This fact would therefore seem to argue that the binding of the acetone is rendered more difficult by the heating of the solution, and that an error in the estimation might arise owing to the fact that the quantity of hypiodite necessary for the formation of iodoform is not forthcoming. In my own experiments I have never been able to find that the circumstance just mentioned plays any part in the estimations, although I have often made use of the supply of iodine in the alkaline iodine solution up to 70–80 % (see *e.g.* experiment 9). Possibly the case is somewhat otherwise with these extremely dilute solutions than with the more concentrated.

Determination of the most suitable time of distillation.

Experiment 3. In the distillation flask are placed 10 cc. of 1 % phosphoric acid and 0.1 cc. of 0.732 % acetone solution. The flask therefore contains 73.2γ acetone. The alkaline iodine solution is introduced into the test-tube in the usual way, 2 cc. of N/200 iodine solution being used. The experiment is repeated eight times, with variations in the time of distillation, the latter being reckoned from the point at which the rattling sound begins.

Table I.

Time of distillation in min.	Acetone in γ
$\frac{1}{4}$	55.1
$\frac{1}{2}$	64.5
$\frac{3}{4}$	68.0
1	72.8
$1\frac{1}{4}$	74.3
$1\frac{1}{2}$	72.8
$1\frac{3}{4}$	74.8
2	73.8

The experiment shows that after 1 minute practically all the acetone has already been distilled over.

Experiment 4. A similar experiment, with the use of 168.6 γ acetone in the flask and 2 cc. of *N*/100 iodine solution.

Table II.

Time of distillation in min.	Acetone in γ
$\frac{1}{2}$	143.9
$\frac{3}{4}$	161.4
1	169.1
$1\frac{1}{2}$	170.8
2	168.1
$2\frac{1}{4}$	168.6
$2\frac{1}{2}$	173.0

Here too the acetone has passed over quantitatively after 1 minute.

The experiment shows that a time of 90 seconds is sufficient for the distillation of pure solutions. In distilling blood samples it is necessary, on account of the frothing, to boil somewhat more carefully. I recommend therefore a somewhat longer time—100 seconds—for the boiling.

The dilution of the blood sample with 10 cc. of 1 % phosphoric acid is designed partly to check the frothing in the distillation, and partly to decompose the aceto-acetic acid. The dilution is carried out in the proportion of 1 : 100. In the distillation about 4 cc. pass over, so that afterwards the dilution is in the proportion of about 1 : 60. This dilution is more than ten times as great as that recommended by Embden for the urine distillation. There can therefore be no reason to suppose that any disturbing iodine-binding substances will, through the concentration due to the distilling process, be made to pass over into the distillate. A dilution of the sample with water while distillation is in progress is superfluous.

3. *The titration.*

After the distillation the tube should stand for 1 minute in the cooler. After a further period of 3 minutes the formation of iodoform is finished. This is shown by the two following experiments.

Experiment 5. To each of six tubes containing 3 cc. of sodium hydroxide and 2 cc. of *N*/200 iodine solution is added 1 cc. of an acetone solution containing 47.4 γ per cc. The first tube is acidified immediately, the second after 1 minute, the third after 2 minutes, and so on. The results of the titration are shown by the following table.

Table III.

Time in min.	Acetone in γ
0	22.5
1	36.3
2	45.5
3	47.4
5	47.6
20	47.4

The first column shows the time that elapsed between the adding of acetone to the alkaline iodine solution and the addition of acid.

In this experiment therefore the iodine is already bound after 3 minutes.

Experiment 6. To each of four flasks, containing 10 cc. of 1 % phosphoric acid, 0.1 cc. of acetone solution containing 53.2 γ acetone was added. Distillation was then carried out in the usual way. The test-tubes were allowed to stand in the cooler for 1 minute. The first tube was acidified immediately after this, the second after 2 minutes, and so on.

Table IV.

Time in min.	Acetone in γ
0	52.6
2	53.3
15	52.2
20	52.2

The experiment shows that the mixture may be acidified 3 minutes after the tube is taken out of the cooler.

After the acidification and when the solution has been sufficiently stirred with the glass rod, starch may be added and titration carried out.

Experiment 7. To each of five tubes containing alkaline iodine solution was added 1 cc. of an acetone solution containing 68.8 γ acetone per cc. When the tubes had stood for 5 minutes they were acidified with sulphuric acid. The contents of the first tube were titrated immediately, those of the second after half a minute, and so on.

Table V.

Time in min.	Acetone in γ
0	67.4
$\frac{1}{2}$	67.4
1	67.4
10	67.9
30	66.9

The first column shows the time that elapsed between the addition of sulphuric acid and the titration.

It is of a certain importance that the titration should be carried out in the test-tubes, partly because the loss of colour can be more clearly observed there than in flasks and beakers, and also because the risk of evaporation of the iodine is then quite inconsiderable. Titrations in flasks and beakers always require a somewhat smaller consumption of thiosulphate than titrations in test-tubes. An iodine solution which, when titrated in a test-tube, used up 1.98 cc. of $N/200$ thiosulphate, used in a 50 cc. flask 1.95 cc., and in a beaker, after 5 minutes' gentle stirring, only 1.40 cc.

The diminution which appears on distillation in the titer of the iodine generally reaches a degree corresponding to 0.03–0.05 cc. of $N/200$ iodine solution. Probably this diminution is due to several causes.

In the text-books of quantitative analysis stress is laid upon the fact that the quantity of iodine demanded for the production of the blue colour will vary considerably, according to the concentration of the iodide present. Care must therefore be taken that the analyses are carried out as far as possible in equal volumes of fluid. With the use of $N/10$ iodine solution however the error becomes so small that it may as a rule be neglected. If $N/100$ iodine solution be used the error may become very great. Treadwell [1913] therefore carries out the following experiment.

To different quantities of water were added 1.5 cc. of starch solution, and $N/100$ iodine solution until the first perceptible blue coloration appeared.

Water in cc.	$N/100$ iodine solution in cc.
50	0.15
100	0.30
150	0.47
200	0.64

When, on the other hand, there was added to the solutions 1 g. of potassium iodide, the consumption of iodine was reduced to about 0.04 cc. up to a volume of 200 cc., which required 0.14 cc. of iodine solution.

In the micromethod the volume of fluid is about 12 cc. and varies within very narrow limits. I have not been able to find that the result of the titration is changed by adding potassium iodide immediately before titrating. Probably sufficient quantities are already present.

The reason for the change in the titer of the iodine solution is probably to be sought in part in another direction. The consumption of iodine becomes greater if, in distilling, the boiling is prolonged beyond the time prescribed. It is greater when a rubber stopper is used as connection between flask and distillation tube than with the use of cork, and is least of all with only glass connections. Finally we find a diminution in the titer of the iodine as soon as we pass 100 cc. of air free of dust through the alkaline iodine solution.

The determination of the titer can however be carried out with perfectly satisfactory accuracy by means of the two blind tests, provided only that the titer of the thiosulphate is quite correct. The result shows that the titer remains sufficiently constant even during very long series of experiments. It is however of great importance for the obtaining of good results that the purest possible chemicals should be used. Only the sodium hydroxide prepared from the metal is of perfectly satisfactory purity. The other preparations often give, even with the test-sample without distillation, too great a consumption of iodine. Ordinary distilled water (according to recent investigations in connection with the salvarsan treatment) often contains important quantities of organic substances which may of course bind iodine. It has been proved also that newly-distilled water gives considerably more even results than ordinary distilled water. I have therefore, in the preparation of all the solutions required by the method, myself distilled the water in a glass distillation apparatus¹.

4. : *The standard solutions.*

The preparation and conservation of these is effected by means of the methods that will be found in most of the text-books of analytical chemistry².

¹ A very convenient apparatus of this kind has been constructed by Hamner and may be obtained from Grave, Stockholm.

² Naturally there can here be no question of the preparation of $N/200$ iodine solution according to Bang by the addition of titrated acid to an excess of iodate and potassium iodide. For when the solution after the distillation is mixed with excess of acid, there is liberated a further quantity of iodine corresponding to the excess of acid.

I have found it safest and most convenient to prepare every day from well-determined $N/10$ iodine and thiosulphate solutions new solutions of $N/100$ and $N/200$ respectively. We have a control of these in the blind tests. The standard solutions should be tested at least once a week. This is most easily done by first testing the thiosulphate solution with bichromate and potassium iodide in a solution acidified with sulphuric acid.

5. Accuracy of the method.

The two following experiments are designed to illustrate the accuracy of the method in the estimation of the amount of acetone in pure solutions before and after the distillation.

Experiment 8. In 12 test-tubes were measured out 3 cc. of $N/2$ sodium hydroxide + 2 cc. of $N/200$ iodine solution + 3 cc. of freshly distilled water. From an acetone solution containing 73.1γ acetone per cc. 0.1 cc. was measured off (with a 1 cc. measuring pipette) in the first tube, 0.2 cc. in the second, and so on; 1 cc. in the tenth tube therefore. The eleventh and twelfth tubes served as blind tests for the determination of the titer of the iodine. After 5 minutes 3.5 cc. of $N/2$ sulphuric acid was added to each of the tubes. After stirring with a glass rod titration was immediately carried out. The following table and Fig. 2 show the results of the titration.

Table VI.

	Calculated	Found (γ)
1	7.3	10.0
2	14.6	15.4
3	21.9	20.8
4	29.2	30.6
5	36.6	37.8
6	43.9	44.9
7	51.2	51.3
8	58.5	61.0
9	65.8	66.8
10	73.1	73.9

The table shows the titration results in the estimation of pure acetone solutions without previous distillation. The second column gives the calculated quantity of acetone, the third column the quantity found by the estimation.

Fig. 2 illustrates this experiment.

Experiment 9. In each of 13 flasks 10 cc. of 1 % phosphoric acid was measured off. Into ten of these flasks rising quantities (from 0.1–1.0 cc.) of an acetone solution containing 73.1γ acetone per cc. were measured off with a 1 cc. measuring pipette. Distillation was then carried out in the usual way, with two blind tests before, and one blind test after, the acetone tests. In the test-tubes 3 cc. of $N/2$ sodium hydroxide + 2 cc. of $N/200$ iodine solution. The acetone values when plotted as in Fig. 2 fall on a straight line.

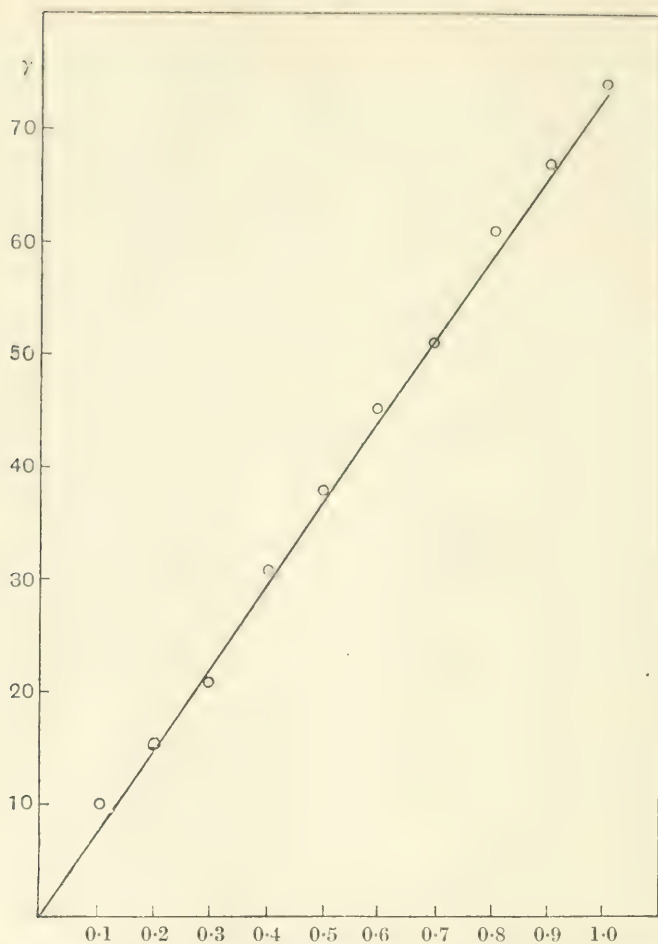


Fig. 2. The calculated values lie upon the oblique line, the values found are marked by circles. Abscissa: the volume of acetone solution added. Ordinate: acetone γ . Estimations without distillation according to experiment (8).

Table VII shows the titration results in an estimation of pure acetone solutions after distillation. Arrangement as in the previous table.

Table VII.

	Calculated	Found (γ)
1	7.3	11.1
2	14.6	16.0
3	21.9	21.8
4	29.2	29.5
5	36.6	38.0
6	43.9	44.0
7	51.2	50.8
8	58.5	57.1
9	65.8	66.2
10	73.1	73.0

The mean error of the single estimation in an estimation of pure acetone solutions appears from the following series.

Experiment 10. Ten estimations were carried out on a pure acetone solution containing 7.32γ per 0.1 cc. The acetone was measured out with a 0.1 cc. pipette. Distillation as usual. In the test-tubes 3 cc. of hydroxide + 2 cc. of $N/200$ iodine solution. The following values were obtained¹:

Acetone in γ : 9.7, 7.7, 7.3, 7.1, 7.3, 7.5, 7.3, 7.3, 8.0, 6.8.

Average: 7.6, $E = \pm 0.805$, $e = \pm 0.255$.

Experiment 11. Ten estimations on an acetone solution containing 57.1γ per 0.1 cc. Estimations carried out as before.

Acetone in γ : 56.7, 56.2, 56.6, 56.7, 56.7, 56.2, 56.0, 57.9, 53.7, 55.7.

Average: 56.24, $E = \pm 1.071$, $e = \pm 0.339$.

From these two experiments it follows that the mean error of the separate estimation hardly exceeds 1γ . The absolute value of the error is about the same, independently of the amount of the acetone. In the first series the error expressed as a percentage of the mean is 10.5% , and in the second series 1.9% .

In the first series the mean is somewhat higher than the calculated value, in the second series somewhat lower. I have not been able to find—provided that sufficient attention is paid to the estimation of the consumption of iodine in the blind experiment—that the method has any appreciable constant error. In general, however, the values are a little too low (about 1γ). This is especially the case if a distillation tube is used having a point with a wider opening than that mentioned in the description of the apparatus. Under such circumstances it may happen that in the distillation the air which passes out before boiling is set up is not completely freed from its acetone in passing through the alkaline solution. Further it is necessary to provide carefully that the glass connection shall close quite tightly. It is advisable to damp the ground glass with a drop of distilled water when the glass tube is fitted into the flask. If a cork connection is used the same cork should not be used too long, but should frequently be replaced by a new one.

It is evident that the method is not suitable for estimations of the acetone percentage in the normal blood. For this 0.1 cc. of blood is too small a quantity. The estimation of the concentration in normal blood falls entirely within the limits of error of the method. In an estimation of normal blood we therefore regularly get titration results which are identical with those of the blind test, or at most show 0.01–0.02 cc. less $N/200$ thiosulphate consumption than these.

The following experiment shows the mean error of the separate estimation in a determination of the amount of acetone in calves' blood, to which acetone was added:

Calculated γ	79.6	39.8	31.8	19.9	15.9	8.0
Found γ	77.1	39.9	32.7	18.9	14.4	8.0
	77.1	39.4	30.3	19.4	15.4	8.0
	77.0	41.3	30.7	19.6	15.9	7.5
	76.7	39.4	30.3	19.1	16.8	7.5
	73.8	40.4	32.2	19.8	16.1	7.7
Average	76.3	40.1	31.2	19.4	15.7	7.7

¹ By E , here and in the following experiment, is meant the mean error of the single estimation; by e the mean error of the mean.

It is my impression that as a rule we are rather more likely to get too low values than too high. In order to illustrate the agreement between the two-fold estimations in the determination of the total acetone concentration in the blood of *diabetics* with acidosis, I give the following report of all the estimations in a case. This series is intended to demonstrate the clinical practicability of the micromethod. The samples of blood were taken by the Hospital laboratory nurse, the distillation was performed by my own laboratory assistant, and finally I myself carried out the titrations.

Table VIII.

Date	Acetone ‰	
	I.	II.
24. i.	0.302	0.321
25. i.	0.334	0.334
26. i.	0.310	0.314
27. i.	0.189	0.184
28. i.	0.114	0.111
29. i.	0.080	0.082
30. i.	0.116	0.097
31. i.	0.264	0.276
1. ii.	0.165	0.160
2. ii.	0.172	0.174
3. ii.	0.126	0.102
4. ii.	0.109	0.114
5. ii.	0.097	0.097
6. ii.	0.106	0.087
7. ii.	0.106	0.087
8. ii.	0.068	0.073
9. ii.	0.204	0.216
10. ii.	0.174	0.174
11. ii.	0.135	0.135
12. ii.	0.094	0.082

In order to show that in the short period of distillation there is time for all the aceto-acetic acid present to be decomposed, a few analyses of the total acetone concentration in urine have been made. In these the total acetone has been estimated by the method of Embden and Schliep and the micro-method simultaneously. The results are shown by the following table.

Table IX.

Subject	Macromethod	Micromethod
A. S.	0.198	0.208 } 0.203 } 0.206
A. S.	0.397	0.392 } 0.375 } 0.385
Mrs L.	1.083	1.060
H. S.	0.329	0.314 } 0.315 } 0.315
H. S.	0.295	0.305 } 0.300 } 0.303

The experiments show that with distillation by the micromethod aceto-acetic acid is decomposed to the same extent as when the macromethod is used.

As we saw in regard to the blood estimations, so also it is impossible to estimate the normal acetone percentage in the urine with any claim to accuracy by the micromethod. As an average figure for the quantity of total acetone present in normal urine may be given approximately 20 mg. per daily quantity of urine. Assuming a daily quantity of 1500 cc. the quantity of acetone present in 0.1 cc. is therefore only a little more than 1γ. The estimation of this quantity falls entirely within the limits of error of the method, which fact was also shown by series of estimations.

Finally it should be emphasised that we do not yet know with perfect certainty whether the values obtained by estimating by means of the iodoform process correspond in every respect to the acetone concentration in question. The possibility is still open that besides acetone other volatile iodoform-producing substances pass over into the distillate in an estimation of the blood and urine of diabetics. Such substances may in general be characterised as having the group CH_3C united with oxygen.

The presence of *ethyl alcohol* in the blood of diabetics demands special attention for the reason that these patients are often given a certain amount of wine in their diet.

However the presence of ethyl alcohol in the blood has no effect upon the results of titration.

Experiment 12. Subject: the author. At 2.15 p.m. on April 15, 1917, 50 cc. of whisky were taken $2\frac{1}{2}$ hours after a meal. This quantity contains about 25 cc. of absolute alcohol (and about 9 mg. of aldehyde, reckoned as acetaldehyde). Before the spirit was drunk two blood samples were taken, and a double sample was also taken 25 and 45 minutes respectively after drinking.

Iodine solution consumed in the blind tests: 1.930 and 1.935 cc. $N/200$ thiosulphate. The iodine solution consumed in the blood tests corresponded to the following quantities of $N/200$ thiosulphate:

Table X.

Blood test.		
Before the taking of the alcohol	1.940	1.940
At 2.40 p.m. (25 min. after taking)	1.940	1.940
At 3.0 p.m. (45 min. after taking)	1.940	1.930

In this experiment the alcohol concentration in the blood during the time taken for the last two tests certainly exceeded 0.5 ‰ notwithstanding the fact that no change in the titer of the iodine can be observed in the tests.

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XXXIX. STUDIES ON THE CYCLOCLASTIC POWER OF BACTERIA.

PART I. A QUANTITATIVE STUDY OF THE AEROBIC DECOMPOSITION OF HISTIDINE BY BACTERIA.

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From the Biochemical Laboratory, Cambridge.

REPORT TO THE MEDICAL RESEARCH COMMITTEE.

(Received November 10th, 1919.)

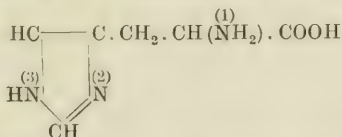
THE investigation of the products formed during the decomposition of histidine by micro-organisms has led to the isolation of a few compounds of biochemical interest. Ackermann [1910] obtained a 42 % yield of histamine (β -iminazolyethylamine) as a result of the growth of mixed putrefactive bacteria on solutions containing histidine. This discovery became of intense interest when Barger and Dale [1910] showed the remarkable physiological activity of this amine, and identified it as one of the active principles of ergot. More recently, Mellanby and Twort [1912] have observed the formation of this amine by pure cultures of an unnamed bacillus which they isolated from the alimentary canal. Berthelot and Bertrand [1912] also observed that a similar decarboxylation of histidine, resulting in the formation of histamine, is readily accomplished by a bacillus to which they gave the name of *Bacillus aminophilus intestinalis*. In fact, the bacterial method of preparation of histamine is used industrially by Hoffmann, La Roche and Co.

It is stated by Windaus and Opitz [1911] that F. Ehrlich obtained β -iminazolyethyl alcohol by the action of yeast on histidine, but I have failed to find that Ehrlich has published his results.

Of the acids which might arise from histidine, by the action of micro-organisms, only two have been isolated. In the paper previously referred to, Ackermann [1910] obtained, in addition to histamine, a very small amount of β -iminazolypropionic acid. The present writer [1917], working with pure cultures of bacteria of the coli-typhosus group, obtained the unsaturated acid, urocanic acid (β -iminazolyacrylic acid), from histidine. The yield in some cases—in particular with *B. paratyphosus A*—was of the order of 60 % of the histidine employed. Later, in extending this work to organisms of other groups, it was found—more particularly with *B. pyocyaneus*—that, although a considerable amount of ammonia was produced and hence at any rate part of the histidine was decomposed, it was impossible to isolate any iminazole

derivatives, except unchanged histidine, from the culture media. For this reason, it was decided to investigate quantitatively the breakdown of the histidine molecule by bacteria. The results of this investigation are given in this paper.

During this work, the fate of the nitrogen contained in the histidine molecule was the only question studied, since, as will be immediately shown, this enables one to form a good idea not only of the fate of the side chain, but of the iminazole nucleus as well. A consideration of the formula for histidine



indicates that the N (numbered (1)) in the side chain may be easily differentiated from the N (numbered (2) and (3)) in the ring. Van Slyke [1911, 1] has indeed shown that, when histidine is treated with nitrous acid—as in his method for the estimation of amino acid nitrogen—only one-third of the total nitrogen in the molecule is liberated as nitrogen, *i.e.* the amino group in the side chain.

Using this as the basis of the method employed, the following estimations were performed at stated intervals on media containing histidine dissolved in Ringer's solution, and sown with the organism under investigation.

A. Total Nitrogen (by Kjeldahl) gives the sum of the three nitrogen atoms ((1), (2) and (3)) whether present, as at the commencement of the experiment, as histidine, or, as in the later stages, as products of the breakdown of histidine.

B. Amino Nitrogen (by van Slyke [1912]) gives the nitrogen (1) contained in the amino group of the side chain, thus providing a measure of the rate of breakdown of the amino group.

C. Ammonia Nitrogen gives the whole of the nitrogen, whether of the side chain or nucleus, which is metabolised by the bacteria into ammonia.

By simple calculations the following values can also be determined:

D. Total Non-amino Nitrogen (other than ammonia nitrogen). The difference between the total nitrogen (*A*) and the sum of the amino nitrogen and ammonia nitrogen (*B* + *C*) gives one the *total* non-amino nitrogen present at any stage,

$$\text{i.e. } A - (B + C) = D.$$

In other words, *D* gives one a measure of the rate of degradation into ammonia of the nitrogen contained in the nucleus.

E. Non-amino Nitrogen (other than ammonia nitrogen and the non-amino nitrogen present as unchanged histidine).

Since the ratio of non-amino nitrogen to amino nitrogen in histidine is 2 : 1, the amount of non-amino nitrogen present at any stage as unchanged histidine will be double the amount of amino nitrogen, *i.e.* 2*B*. Thus

$$E = D - 2B.$$

In other words, E gives a measure of the rate of production from histidine of compounds (other than ammonia) containing non-amino nitrogen.

It will be noted that no account is taken of the nitrogen which may be used for synthesis, *e.g.* in the formation of bacterial protein. It is, however, very probable, as is shown from results obtained with *B. proteus*, that the amount of nitrogen used for synthesis by these bacteria is relatively negligible (with the possible exception of *B. pyocyaneus*) and does not vitiate the general conclusions drawn from these experiments.

EXPERIMENTAL.

In all cases, the culture solution was contained in a two litre flask, fitted with a rubber stopper carrying two glass tubes. One of these, bent at an angle of about 60° , was fitted at one end with a plug of cotton wool about three inches long, and the other end was inserted, to a distance of about an inch, through the rubber stopper. The other tube, bent at the same angle, was fitted at one end with a piece of rubber tubing, carrying a small glass tube drawn out to a sealed capillary point. The other end of the tube was inserted through the rubber stopper, and terminated about $\frac{1}{4}$ inch from the bottom of the flask. The apparatus thus closely resembled an ordinary wash bottle, except that the tube carrying the cotton wool plug was bent downwards, to lessen the risk of contamination.

The following solution was then introduced into the flask, and the whole was autoclaved at 120° for an hour.

Distilled water	1550 c.c.
Sodium chloride	16.2 g.
Potassium chloride	0.756 g.
Calcium chloride	0.432 g.
Normal caustic soda	23.2 c.c.

(to neutralise the HCl of the histidine hydrochloride).

In a separate 300 c.c. conical flask, 4.86 g. of pure histidine monohydrochloride was dissolved in 250 c.c. of water, and autoclaved. This separate autoclaving was preferred, as histidine undergoes undesirable changes to a far less extent in acid solution (as the hydrochloride) than in alkaline solution (as the free base).

The contents of the two flasks, after mixing, thus gave a 0.2 % solution of histidine in Ringer's solution, this being the medium previously used [Raistrick, 1917]. In order to introduce the solution of the histidine hydrochloride into the large flask, the following procedure was used. A piece of rubber tubing, fitted with a screw clip, was fixed on the end of the tube containing the cotton wool plug, and, by sucking out a little of the air and then turning the clip, a slight decrease of the pressure of the air inside the flask was obtained. The rubber connection on the other tube was then closed by a clip, and the end of the capillary tube was broken off and carefully sterilised. It was then introduced beneath the surface of the histidine solution which, on opening

the screw clip, passed over into the larger flask. The flask was then sown with an emulsion in sterile Ringer's solution of the organism under investigation, obtained by emulsifying the growth from 20 agar slopes, after 24 hours' incubation. The bacterial emulsion was introduced into the large flask in the same way as the solution of histidine hydrochloride. Samples for analysis were taken from the large flask by creating a slight increase in the pressure of the air in the flask, by blowing gently through the tube containing the cotton wool plug, and then clipping the rubber tube. After closing the rubber connection on the other tube with a clip, the contents of the flask were shaken, and the end of the capillary tube, which was always fused in the Bunsen flame after taking a sample, was broken and sterilised. On opening the clip, the culture media coming over was collected, after rejecting the first 20 or 30 c.c. Care was taken both in sowing the culture media with the organism studied and when samples for analysis were subsequently taken out that no air was allowed to enter the flask except through the cotton wool plug. Bacteriological control showed that, in all the experiments here recorded, there was no contamination. The flasks were incubated at 37°, and, since the tube containing the cotton wool plug was open to the air during incubation, the experiments took place under aerobic conditions.

The following estimations were made on each sample:

I. *Total nitrogen.*

The nitrogen present in 100 c.c. medium was estimated by the Kjeldahl method in the usual way. Since experience showed that there was practically no variation in the total nitrogen during the experiment, determinations were only made at the beginning and end of the experiment. In this, and in the subsequent estimation of free ammonia, the excess of standard acid was always estimated by adding potassium iodide and potassium iodate, and titrating the iodine liberated with standard sodium thiosulphate.

II. *Ammonia and amino acid nitrogen.*

200 c.c. of culture medium was introduced, without previous sterilisation, into a litre Claisen distilling flask, 100 c.c. alcohol, and 10 c.c. of a 10 % suspension of pure lime were added. The free ammonia was distilled off, *in vacuo*, at 40°–45°, and absorbed in an excess of standard *N*/10 acid. The distillation apparatus used was that recommended by van Slyke [1911, 2] for the estimation of the ammonia formed during the hydrolysis of proteins. By this means, one avoids the decomposition into ammonia of readily hydrolysable nitrogen compounds which might be formed by the action of bacteria on histidine (*e.g.* urea). By titration of the excess of acid present, the amount of ammonia formed by the organism studied can be calculated.

After half-an-hour's distillation, the apparatus was disconnected. An excess of glacial acetic acid was added to the Claisen flask, and the solution was concentrated *in vacuo* to a volume of a few c.c. This was then washed out

quantitatively into a 25 c.c. flask, and 2 c.c. of glacial acetic acid were added to prevent any possible action by bacteria which might by this time still be alive, and the whole was made up to 25 c.c. The amino acid nitrogen present was then estimated by van Slyke's [1912] method, 8 c.c. being used for each of the estimations, which were done in triplicate.

All the samples were taken at the same time each day, so that periods represented whole days, and all the estimations on one sample were done on the same day. In the subsequent table, the different forms of nitrogen are expressed as milligrams of nitrogen per 100 c.c. of the medium, and are corrected for impurities due to reagents, etc.

In all the curves given to express graphically the results obtained (pp. 452—455), the following plan has been used:

Curve *B* = ————— represents the amino acid nitrogen (*B*) at different times.

Curve *C* = represents the ammonia nitrogen (*C*).

Curve *D* = - - - - - represents the *total* non-amino nitrogen (*D*).

Curve *E* = — - - — represents the non-amino nitrogen (*E*).

A consideration of the results shows that, with the exception of *B. proteus vulgaris*, the general *type* of curve is very similar in each case. They all show a gradual decrease in amino acid nitrogen (*B*) and total non-amino nitrogen (*D*), along with a gradual increase in the ammonia nitrogen (*C*) and non-amino nitrogen (*E*). Notably with *B. pyocyaneus*, and to a less extent with *B. proteus vulgaris*, the end point of the reaction is reached much more quickly than with the other organisms. A comparison of the relative *rates* of production of ammonia nitrogen (*C*) and non-amino nitrogen (*E*), and of the decomposition of amino acid nitrogen (*B*) and total non-amino nitrogen (*D*) shows the following interesting differences:

(a) With *B. paratyphosus A*, and the two experiments with *B. paratyphosus B*, the *rates* of each of these reactions increases to a maximum which occurs between the twentieth and thirtieth days, and then decreases.

(b) With *B. faecalis alcaligenes*, *B. pyocyaneus*, and *B. proteus vulgaris*, the *rates* of each of these reactions are all decreasing over the whole course of incubation.

B. proteus vulgaris is sharply differentiated from all the other organisms studied, by the fact that no ammonia is produced from the nitrogen contained in the iminazole nucleus.

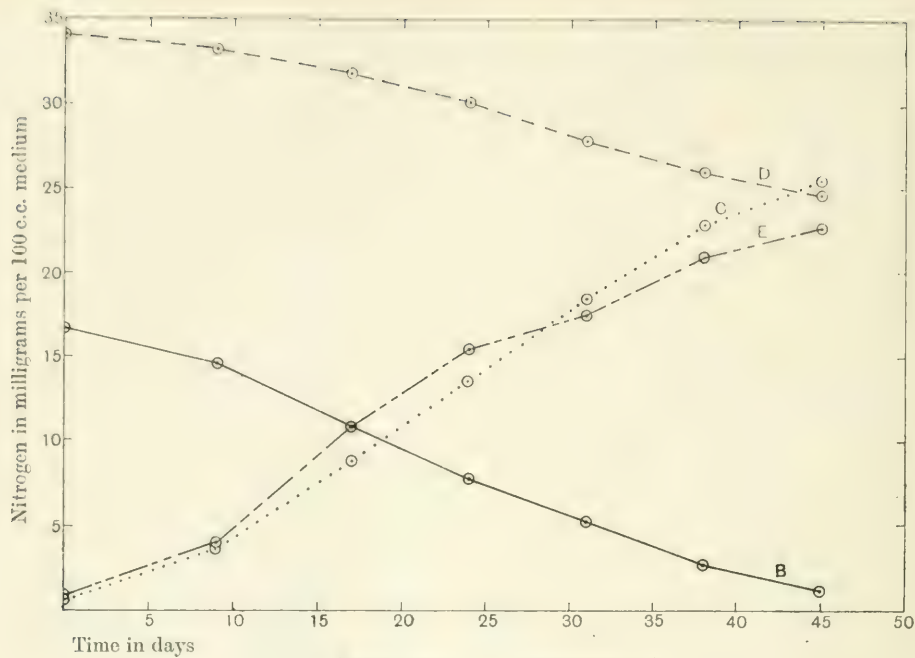
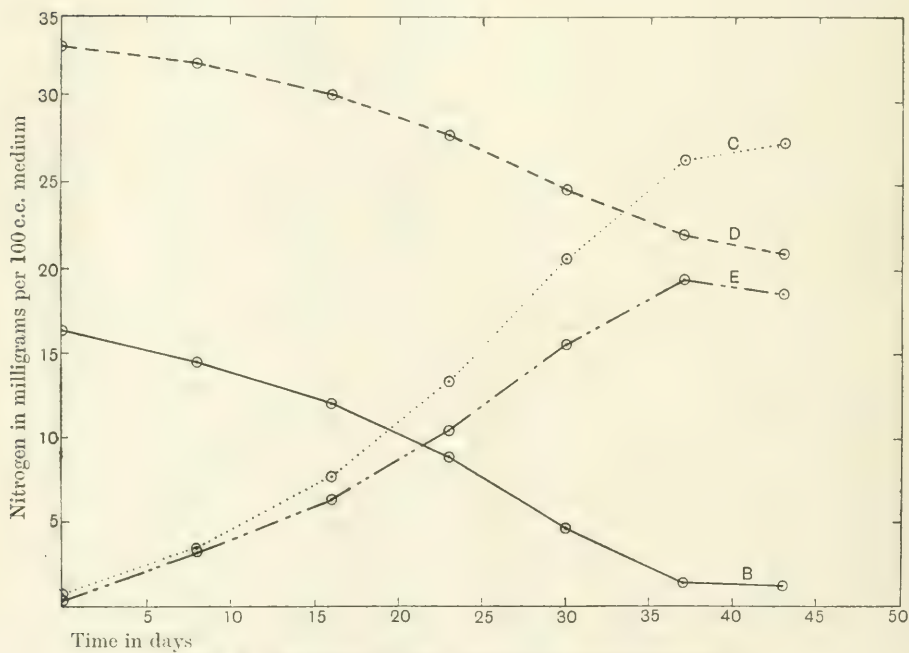
A clearer conception of the meaning of the results obtained is gained by comparing the ratios of values obtained by experiment with those which would arise in any hypothetical case. Thus, if the following values are assigned to the letters given:

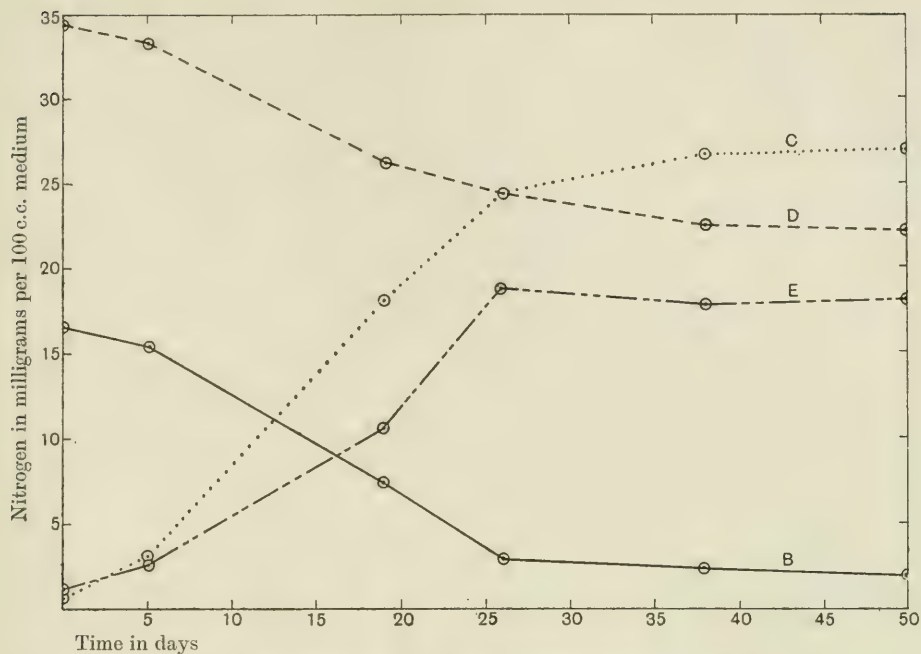
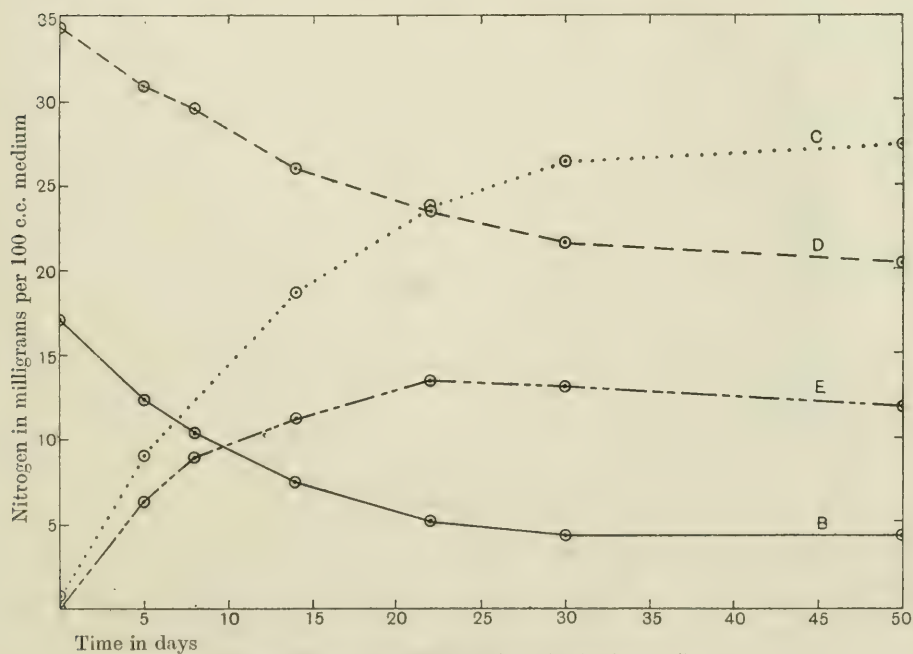
B = Total decrease in amino acid nitrogen during the course of the experiment, *i.e.* the difference between the initial and final amino acid nitrogen values;

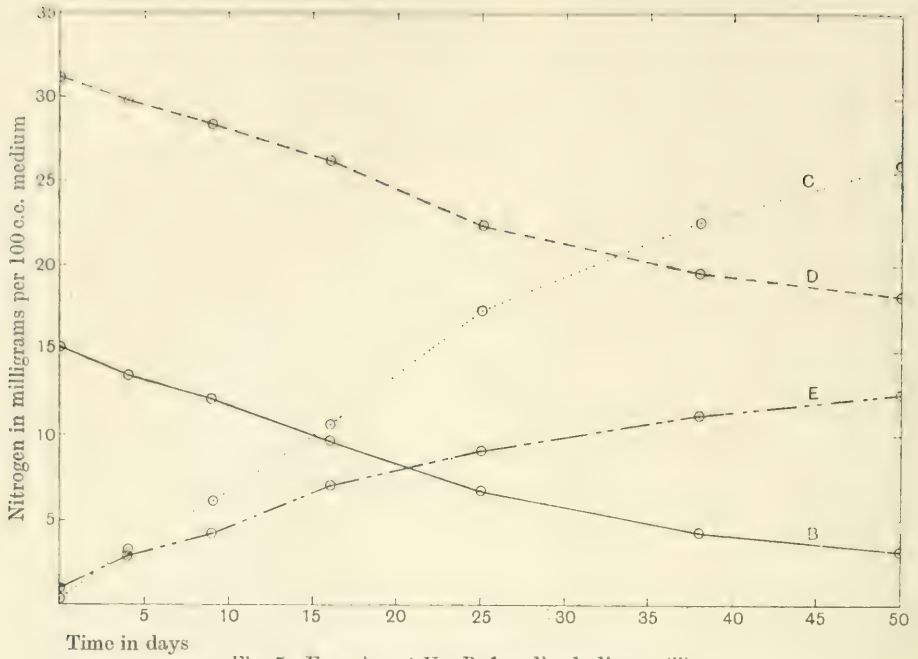
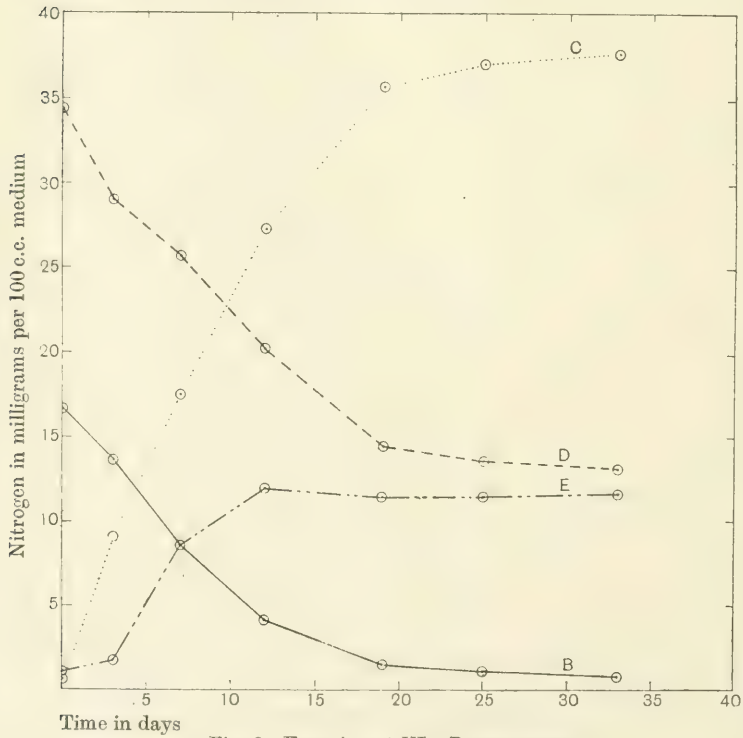
Table I.

Distribution of nitrogen in milligrams per 100 c.c. of medium.

Organism studied	Period of incubation in days	Total nitrogen A	Amino acid nitrogen B	Ammonia nitrogen C	Total non-amino nitrogen D	Non-amino nitrogen E
<i>B. paratyphosus</i> A	—	51.41	16.70	0.59	34.12	0.72
Expt I (Fig. 1)	9	51.41	14.65	3.53	33.23	3.93
	17	51.41	10.82	8.75	31.84	10.82
	24	51.41	7.72	13.51	30.18	15.44
	31	51.41	5.17	18.42	27.82	17.48
	38	51.41	2.58	22.83	26.00	20.84
	45	51.20	1.02	25.49	24.69	22.65
<i>B. paratyphosus</i> B (Birmingham)	—	50.07	16.37	0.51	33.19	0.45
Expt II (Fig. 2)	8	50.07	14.47	3.39	32.21	3.27
	16	50.07	12.02	7.70	30.35	6.31
	23	50.07	8.79	13.29	27.99	10.41
	30	50.07	4.62	20.69	24.76	15.52
	37	50.07	1.35	26.55	22.17	19.47
	43	49.75	1.22	27.55	20.98	18.54
<i>B. paratyphosus</i> B (Schottmüller)	—	51.75	16.63	0.67	34.45	1.19
Expt III (Fig. 3)	5	51.75	15.36	3.11	33.28	2.56
	19	51.75	7.33	18.13	26.29	11.63
	26	51.75	2.83	24.46	24.46	18.80
	38	51.75	2.39	26.76	22.60	17.82
	73	51.06	1.40	27.67	21.99	19.19
<i>B. faecalis alcaligenes</i> (i)	—	52.21	17.14	0.66	34.41	0.13
Expt IV (Fig. 4)	5	52.21	12.29	9.02	30.90	6.32
	8	52.21	10.41	12.12	29.68	8.86
	14	52.21	7.44	18.68	26.09	11.21
	22	52.21	5.04	23.68	23.49	13.41
	30	52.21	4.24	26.41	21.56	13.08
	57	52.05	4.25	27.75	20.05	11.55
<i>B. faecalis alcaligenes</i> (ii)	—	46.44	15.13	0.21	31.10	0.84
Expt V (Fig. 5)	4	46.44	13.45	3.26	29.73	2.83
	9	46.44	12.09	6.03	28.32	4.14
	16	46.44	9.62	10.58	26.24	7.00
	25	46.44	6.69	17.35	22.40	9.02
	38	46.44	4.24	22.57	19.63	11.15
	72	47.76	1.07	31.38	15.31	13.17
<i>B. pyocyaneus</i>	—	51.60	16.68	0.55	34.37	1.01
Expt VI (Fig. 6)	3	51.60	13.62	9.02	28.96	1.72
	7	51.60	8.52	17.49	25.59	8.55
	12	51.60	4.14	27.27	20.19	11.91
	19	51.60	1.50	35.65	14.45	11.45
	25	51.60	1.03	37.04	13.53	11.47
	33	51.51	0.75	37.61	13.15	11.65
<i>B. proteus vulgaris</i>	—	51.63	16.62	0.66	34.35	1.11
Expt VII (Fig. 7)	5	51.63	10.77	6.20	34.66	13.12
	8	51.63	8.67	8.38	34.58	17.24
	15	51.63	7.42	9.44	34.77	19.93
	23	51.63	6.73	9.93	34.97	21.51
	35	51.60	5.87	10.59	35.14	23.40

Fig. 1. Experiment I. *B. paratyphosus* A.Fig. 2. Experiment II. *B. paratyphosus* B (Birmingham)

Fig. 3. Experiment III. *B. paratyphosus* B (Schottmüller)Fig. 4. Experiment IV. *B. faecalis alcaligenes* (i)

Fig. 5. Experiment V. *B. faecalis alcaligenes* (ii)Fig. 6. Experiment VI. *B. pyocyaneus*

C = Total increase in ammonia nitrogen under the same conditions;

D = Total decrease (or increase) in total non-amino nitrogen (as previously defined);

E = Total increase in non-amino nitrogen (as previously defined);

the ratios which these values would have in the following hypothetical cases may be considered:

(i) *Simple de-aminisation of the side chain.* If the amino acid nitrogen in the side chain were converted into ammonia, then

$$\frac{C}{B} = 1, \quad \frac{D}{B} = 1, \quad \frac{E}{B} = 1.$$

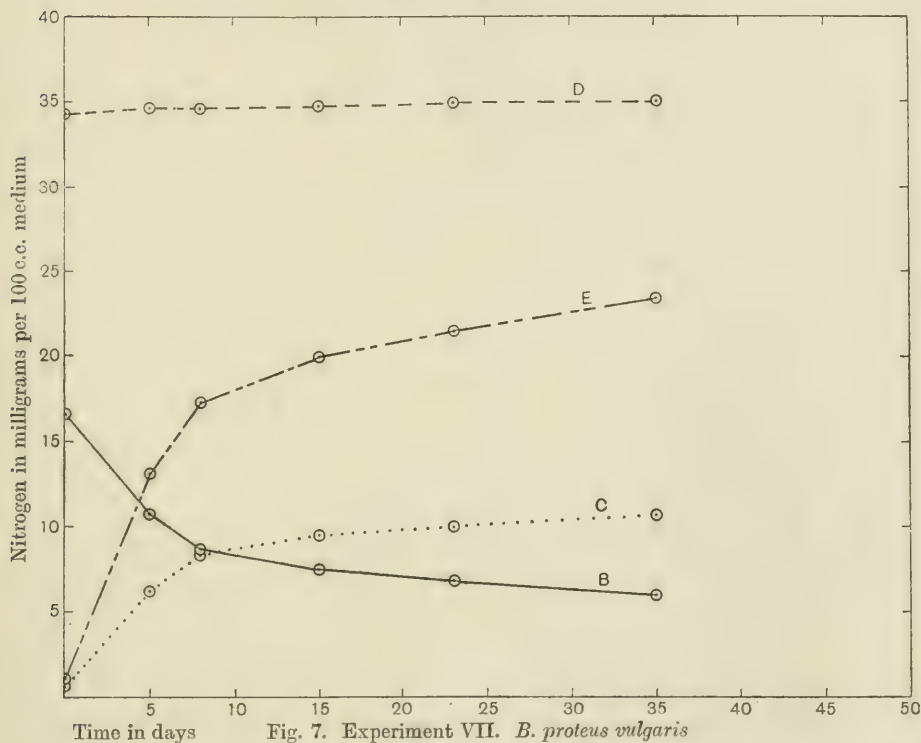


Fig. 7. Experiment VII. *B. proteus vulgaris*

(ii) *Simple decarboxylation of the side chain.* If this reaction alone took place, no ammonia would be formed. The amino acid nitrogen would decrease, since the amine grouping $-\text{CH}_2-\text{NH}_2$ does not give any appreciable amount of nitrogen under the conditions employed for estimating amino acid nitrogen.

Then

$$\frac{C}{B} = 0, \quad \frac{D}{B} = 1, \quad \frac{E}{B} = 3.$$

(iii) *Production of ammonia from both side chain and nucleus.* Since there are two nitrogen atoms in the iminazole nucleus, we may assume that either

(a) both nuclear nitrogen atoms and side chain nitrogen would be attacked at the same rate, then

$$\frac{C}{B} = 3, \quad \frac{D}{B} = 2, \quad \frac{E}{B} = 0.$$

or (b) only one of the nuclear nitrogen atoms, in addition to the side chain nitrogen, would be attacked, then

$$\frac{C}{B} = \frac{2}{1}, \quad \frac{D}{B} = \frac{1}{1}, \quad \frac{E}{B} = \frac{1}{1}.$$

The ratios obtained for the different bacteria investigated, calculated from the results previously given, are summarised in the following table.

[Thus e.g. for *B. paratyphosus* A, $\frac{C}{B} = \frac{25.49 - 0.59}{16.70 - 1.02} = \frac{24.90}{15.68} = \frac{1.59}{1}$.]

Table II.

Organism	$\frac{C}{B}$	$\frac{D}{B}$	$\frac{E}{B}$
<i>B. paratyphosus</i> A	$\frac{1.59}{1}$	$\frac{0.60}{1}$	$\frac{1.40}{1}$
<i>B. paratyphosus</i> B (Birmingham)	$\frac{1.78}{1}$	$\frac{0.81}{1}$	$\frac{1.19}{1}$
<i>B. paratyphosus</i> B (Schottmüller)	$\frac{1.77}{1}$	$\frac{0.82}{1}$	$\frac{1.18}{1}$
<i>B. faecalis alcaligenes</i> (i)	$\frac{2.10}{1}$	$\frac{1.11}{1}$	$\frac{0.886}{1}$
<i>B. faecalis alcaligenes</i> (ii)	$\frac{2.22}{1}$	$\frac{1.12}{1}$	$\frac{0.877}{1}$
<i>B. pyocyaneus</i>	$\frac{2.33}{1}$	$\frac{1.33}{1}$	$\frac{0.67}{1}$
<i>B. proteus vulgaris</i>	$\frac{0.985}{1}$	$\frac{0.073}{1}$	$\frac{2.07}{1}$

From a consideration of the results given in Table II, and from the different graphs, it seems justifiable to draw the following conclusions:

(i) *B. proteus vulgaris* alone, of the organisms investigated, cannot produce ammonia from either of the nitrogen atoms in the iminazole nucleus, although it does so from the nitrogen atom in the side chain. This is probably due to one of two causes:

(a) The inability of the organism to open the ring. If this explanation, which seems the more probable, is correct, the reason would appear to be that the enzyme, which enables the other bacteria investigated to open the iminazole ring, is not present in *B. proteus vulgaris*. The evidence offered in this paper seems to point, in fact, to the presence in many bacteria of a specific enzyme (perhaps more than one), which has the power of splitting open the iminazole ring, and producing ammonia from the nitrogen atoms contained in the opened ring. The ease with which this change is brought about by bacteria is in marked contrast to the well-known stability of the iminazole ring towards most chemical reagents.

(b) The possibility, an improbable one, that although *B. proteus vulgaris* can open the iminazole ring it cannot convert the nitrogen contained in the ruptured ring into ammonia.

(ii) None of the bacteria produced simple decarboxylation of the histidine, and it is to be regretted that for the sake of completeness it was impossible

under conditions prevailing at the time, to obtain a culture of one of the well-known decarboxylating bacteria.

(iii) *B. faecalis alcaligenes*, *B. pyocyaneus*, *B. paratyphosus A*, and *B. paratyphosus B* produce ammonia both from the nitrogen in the side chain and in the nucleus. All these bacteria attack the histidine molecule as a whole by a series of reactions which proceed concurrently, and produce a complete breakdown of at least a portion of the iminazole ring. Two of them, *B. faecalis alcaligenes* and *B. pyocyaneus*, produce ammonia from the ring more quickly than they produce it from the side chain. Since the ratio $\frac{D}{B}$ is greater than $\frac{1}{1}$ and since the ratio $\frac{C}{B}$ is greater than $\frac{2}{1}$ with these two organisms, it follows that ammonia is formed by each of them from each of the three nitrogen atoms in the histidine molecule. The fact that the ratio $\frac{E}{B}$ is in the one case $\frac{0.88}{1}$ and in the other $\frac{0.67}{1}$ probably explains the previously described failure to isolate any iminazole compound from the action of *B. pyocyaneus* on histidine.

A comparison of the ratios obtained with *B. paratyphosus A* and *B. paratyphosus B* shows that these two organisms, and more particularly *B. paratyphosus A*, produce ammonia from the side chain more quickly than they do from the ring since the ratio $\frac{D}{B}$ is in each case less than $\frac{1}{1}$. The fact that the ratio $\frac{E}{B}$ for *B. paratyphosus A* is relatively large ($\frac{1.40}{1}$) explains why such a good yield of urocanic acid was obtained as a result of the action of this organism on histidine, since $\frac{E}{B}$ represents the ratio of end products, other than ammonia, to histidine decomposed.

It is of interest to note that though the value of any one of the ratios $\frac{C}{B}$, $\frac{D}{B}$, $\frac{E}{B}$ is different for each of the different bacteria investigated, they show very good agreement for different strains of the same organism (e.g. for two different strains of *B. paratyphosus B* and of *B. faecalis alcaligenes*).

SUMMARY.

The aerobic breakdown of histidine by bacteria (*B. paratyphosus A*, *B. paratyphosus B*, *B. faecalis alcaligenes*, *B. pyocyaneus*, and *B. proteus vulgaris*) was studied quantitatively.

The results obtained showed that:

(i) *B. paratyphosus A*, *B. paratyphosus B*, *B. faecalis alcaligenes*, and *B. pyocyaneus* can attack, and produce ammonia from, the nitrogen atoms in both the side chain and the iminazole nucleus of the histidine molecule, thus proving that they are able to open the iminazole ring.

(ii) With *B. proteus vulgaris*, ammonia is formed only from the side chain nitrogen, and it is very probable that this organism is unable to open the iminazole ring.

I wish to take this opportunity of expressing my gratitude and indebtedness to Professor F. Gowland Hopkins for much valuable criticism and advice during the progress of this research.

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XL. NITROGEN METABOLISM IN *SACCHAROMYCES CEREVISIAE*.

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INTRODUCTION.

DURING the last few years, the assimilation of nitrogen by the lower organisms of the vegetable kingdom has formed the subject of an ever-increasing number of investigations, the results of which have rendered evident the great importance of this question in its bearing on the problems of both pure and applied chemistry.

Of these researches, not the least suggestive, from a purely chemical, as well as from an industrial, standpoint are those of Felix Ehrlich and his collaborators, who succeeded in establishing the fact that the "fusel oil" of the distiller is produced by the action of yeast on the amino-acids in the wort, yielding the corresponding alcohols, such amino-acids being the degradation products of the proteins originally present.

It is not necessary to trace the history of this theory, first put forward by A. Müller in 1857, and either disregarded or discredited until Ehrlich in 1903 proved that the α -amino-acids when treated with yeast in a saccharine solution give the corresponding alcohols.

The object of the work described in the present communication was the closer investigation of those factors which affect the nitrogen metabolism in yeast, not only from the point of view of the assimilation of the nitrogen detached from the amino-acid during its conversion into an alcohol, but also from the point of view of an apparent excretion of nitrogen which takes place with yeast under certain circumstances.

PART I. INVESTIGATION OF THE FACTORS WHICH INFLUENCE THE ASSIMILATION OF NITROGEN FROM AMINO-ACIDS BY YEAST.

Theoretical.

In view of the important and fundamental character of the work of Ehrlich, it was deemed advisable to repeat the final experiments of this

worker, and the results obtained with leucine confirmed Ehrlich's conclusions, namely, (1) that the amount of nitrogen lost by the amino-acid equals the amount gained by the yeast; (2) that the amount of nitrogen lost by the liquid corresponds to the amount of amyl alcohol formed; (3) that the amount of alcohol formed corresponds to about half the weight of leucine introduced initially into the solution. An extension of the same method to the desamination of asparagine gave further confirmatory results, but here, as would be expected, analysis of the resultant liquid failed to reveal the presence of any of the higher alcohols, a result quite in agreement with the theory of Ehrlich, according to whose equations the only product formed by the extraction of nitrogen from asparagine would be ethyl alcohol.

The main conditions under which these experiments were carried out may be briefly summarised as follows:

- (1) Yeast of low nitrogen content.
- (2) Non-multiplying yeast.
- (3) Large amount of sugar available for fermentation.

The first of these conditions is contingent on the statement of Ehrlich that a yeast of low nitrogen content has a greater assimilatory power for nitrogen than has one in which the percentage of protein matter is high, and a comparison of two yeasts of the same race but containing different percentages of nitrogen showed that there was a small but distinct difference between the amounts of nitrogen assimilated by the yeasts, the comparison being made with yeasts in which the difference in the nitrogen content was artificially induced by previous treatment.

The amount of yeast added to the saccharine amino-acid solution in Ehrlich's experiments precluded the possibility of cell reproduction and it consequently appeared probable that the conclusions drawn from these experiments might not be valid if the yeast were in a state of active cell multiplication during the time the nitrogen assimilation was taking place.

To elucidate this point saccharine amino-acid solutions containing the inorganic salts commonly employed as yeast nutriment (Pasteur) with the exception of the ammonium salts—the amino-acid taking their place—were seeded with a pure culture of *S. cerevisiae* in such amounts that the yeast concentration in three experiments was 1, 10, and 100 cells per unit volume respectively, and the results so obtained are of a most interesting character.

In the first place they show that an excess of yeast extracts the maximum total amount of nitrogen, that is to say, that, from equal volumes of an amino-acid solution a suspension of 100 cells/ T^1 assimilates a much greater amount of nitrogen than a suspension of 10 cells/ T . On the other hand, regarded from the point of view of a single cell, the greater the cell concentration the less the amount of nitrogen assimilated by each cell (calculated on the number of cells found in the solution at the end of the experiment).

¹ T is the symbol for the standard unit volume of 1/4000 cubic millimetre.

The following figures (mean of three typical experiments) demonstrate this:

Table I.

Rate of seeding	G. nitrogen extracted per unit cell	
	Leucine	Asparagine
100 cells/ <i>T</i>	3.37×10^{-4}	11.53×10^{-4}
10 cells/ <i>T</i>	8.87×10^{-4}	12.85×10^{-4}
1 cell/ <i>T</i>	31.95×10^{-4}	31.45×10^{-4}

The figures (see experimental section) show a noticeable lack of increase in the number of cells in those experiments seeded at the rate of 1 cell/*T*.

There are apparently three factors which might account for this, (1) lack of oxygen, (2) effect of the products of fermentation, (3) effect of some substance originally present.

Experiments proved the first factor to be of no practical importance, for aerated and non-aerated fermentations yielded similar results.

The second factor is more difficult of consideration. On the assumption—a very simple one—that the decrease in the amount of nitrogen is an indication of the amount of action which has taken place, and that consequently it is proportional to the quantity of any inhibitant formed, it follows that (taking concrete examples from the results recorded in the experimental part) in two leucine experiments (Tables III and IV) seeded with 10 (*B*) and 1 (*C*) cell per unit volume respectively, the inhibitant should be in the ratio 0.0317 to 0.0195, a concentration reached when the number of cells in these two experiments were 32 and 7 respectively. Thus it follows that each cell in *B* would be influenced by 0.0317/32 parts of inhibitant whilst each cell in *C* had to sustain the action of 0.0195/7 parts, that is 9.9 parts in *B* to 28.0 parts in *C*. Therefore if such a body is formed there is here a reason for the lack of increase in *C*, the experiment seeded with 1 cell per unit volume. It should be noted, however, that careful analysis of the fermented liquids failed to show the presence of any inhibitory body.

With regard to the third factor it must be remembered that amino-acids although providing food for yeast, exert retarding influences on the reproductive capacity of the yeast cell, and it may also be that these amino-acid preparations may contain minute traces of bodies which in the light of modern knowledge may have an inhibitory effect on the reproductive activity of the yeast. It has been shown that in the case of antiseptics, a certain concentration will stop the growth of an approximately definite number of organisms, but if the number of organisms be increased there will be a limiting value for the amount of antiseptic per cell, below which value reproduction can take place. It may be that the preparations of amino-acid used act in a similar manner, and that 100 cells/*T* gives in the above experiments a concentration of amino-acid below the limiting value, while the 1 cell/*T* is above this value and consequently reproduction is retarded.

Yet one other point of interest is attached to this series of experiments, namely, the irregularity of the final nitrogen coefficient of the yeast; but a comparison shows that the final value of this coefficient is apparently connected in some way with the reproductive activity of the yeast, and that for one set of factors the yeast has apparently a specific nitrogen coefficient. (Table VIII, p. 468.)

Experimental.

1. *Fermentation of leucine and asparagine solutions.* The preliminary experiments which were carried out during the course of these investigations were, as has already been stated, conducted along the lines given by Ehrlich himself, and consisted essentially of the fermentation of sugar solutions containing amino-acids, by yeasts of low nitrogen content. The results demonstrated clearly the truth of the conclusions which this worker himself drew. Applying the same method of experiment to the fermentation of asparagine, further confirmatory results were obtained, but in this case, as would be expected, careful analysis of the resultant liquid failed to show the presence of any higher alcohol.

2. *Effects of original nitrogen coefficient of yeast.* In order to test the statement that a yeast of low protein content extracts more nitrogen than one in which the amount of protein is high, a comparison was made between yeasts of different protein content. The seed-yeasts were prepared, in the one case by allowing the yeast to ferment without cell-multiplication in a wort rich in nitrogen, and in the other case by growing a sample of the same yeast in the wort diluted with water, the yeast being seeded at the rate of 10 cells per unit volume *T*. The yeasts thus prepared were finally washed and introduced at the rate of 10 cell/*T* into a 10 % cane sugar solution containing 0.1 % nitrogen as asparagine, and the liquid maintained at 37°. At the end of two days the fermentation was finished, and the following results were obtained.

Table II.

Seed yeast		Total nitrogen in solution		% nitrogen assimilated
Condition	% nitrogen	Before	After	
Low	6.85	0.1000	0.0748	25.20
High	9.32	0.1000	0.0780	22.00

The distinct, although small, difference between the two numbers in the final column confirms Ehrlich's statement that the amount of nitrogen extracted is to some extent related inversely to the amount of protein originally present in the organism. At least this is so for yeasts of an *artificially* high or low nitrogen content.

3. *Effects of cell reproduction on nitrogen extraction.* The effect of active cell multiplication on nitrogen absorption was investigated by seeding a nutrient amino-acid solution with a pure culture of *S. cerevisiae*, the yeast being added to the solution as a suspension in sterile water, in such volume

that the resultant mixture contained in three series, 1, 10, and 100 cells/*T*. The composition of the nutriment itself was:

Cane sugar	10.00 %
Potassium di-hydrogen phosphate	0.20
Calcium phosphate	0.02
Magnesium sulphate	0.02
Nitrogen (as leucine or asparagine)	0.10
Water to	100.00

Tables III and IV give the results obtained in two such series.

Table III.

Source of N	Rate of seeding per unit volume	Before fermentation			After fermentation			Difference	
		<i>a</i> Total N	<i>b</i> N in yeast	<i>c</i> N in acid	<i>d</i> Total N	<i>e</i> N in yeast	<i>f</i> N in acid	<i>g</i> Increase N in yeast	<i>h</i> Decrease N in acid
Leucine } Aspar. }	100	{ 0.3256 0.3991	{ 0.2122 0.1764	{ 0.1134 0.2227	{ 0.3227 0.3976	{ 0.2444 0.2830	{ 0.0783 0.1146	{ 0.0322 0.1066	{ 0.0351 0.1081
Leucine } Aspar. }	10	{ 0.1346 0.2403	{ 0.0212 0.0176	{ 0.1134 0.2227	{ 0.1280 0.2378	{ 0.0463 0.0561	{ 0.0817 0.1817	{ 0.0251 0.0385	{ 0.0317 0.0410
Leucine } Aspar. }	1	{ 0.1155 0.2245	{ 0.0021 0.0018	{ 0.1134 0.2227	{ 0.1206 0.2254	{ 0.0267 0.0280	{ 0.0939 0.1974	{ 0.0246 0.0262	{ 0.0195 0.0253

Table IV.

Source of N	Rate of seeding per unit volume	No. of cells per <i>T</i>				N coefficient of yeast $\times 10^4$			N increase per unit cell $\times 10^4$
		<i>k</i> Before	<i>l</i> After	<i>l/k</i> Coefficient of multiplication	<i>m</i> No. of new cells	<i>n</i> Before	<i>p</i> After	<i>q</i> Difference	
Leucine } Aspar. }	100	{ 97.8 97.8	{ 94.3 93.4	{ — —	{ — —	{ 21.7 18.0	{ 25.9 30.3	{ + 4.2 + 12.3	{ 3.41 11.50
Leucine } Aspar. }	10	{ 9.8 9.8	{ 32.0 33.1	{ 3.28 3.37	{ 22.2 23.3	{ 21.7 18.0	{ 14.5 17.0	{ - 7.2 - 1.0	{ 7.84 11.60
Leucine } Aspar. }	1	{ 1.0 1.0	{ 6.9 7.9	{ 6.90 7.90	{ 5.9 6.9	{ 21.7 18.0	{ 38.7 35.5	{ + 17.0 + 17.5	{ 35.65 33.16

In the above results the small increase in the number of cells in those experiments seeded at the rate of 1 cell/*T* is very noticeable, and the following experiments were conducted in order to get some idea of the state of the cells at the termination of the fermentation.

4. *Final condition of the yeast cell in experiments seeded at 1 cell/T.* A nutrient asparagine solution (of composition given above) was seeded at the rate of 1 cell/*T*, and allowed to ferment at 37° in the usual manner. At the conclusion of the fermentation, a count of the number of yeast cells was made, the yeast separated by filtration and the filtrate distilled *in vacuo* at 40°. The residue was then dissolved in 10 % sucrose solution, and the solution made up to the original volume of the asparagine solution, and finally treated with the previously separated yeast. On the completion of the second fermentation, a count of the cells was again made, with the following results:

Table V.

Fermentation	No. of cells per unit volume		Coefficient of multiplication
	First	Second	
First	1.10	9.20	8.36
Second	9.00	31.40	3.49

This then conclusively proves that the yeast in the initial experiment (seeded at the rate of 1 cell/*T*) was not killed, but that its *reproductive activity had been retarded*.

5. *Effect of aeration on nitrogen extraction by yeast.* Fermentations were conducted as previously described (leucine and asparagine being added as the sources of nitrogen in two parallel series), except that the fermenting liquid was aerated by the passage of a stream of sterile air for a short time each day. The results are given in the following table, together with those previously shown in Table III for purposes of comparison. Experiments with asparagine gave confirmatory results.

Table VI.

Conditions		Nitrogen in solution g. per 100 cc.			No. of cells per unit volume			Coefficient of multiplication
Rate of seeding	Aeration	Before	After	Decrease	Before	After	Increase	
100	{ Non-Aerated	0.1134	0.0783	0.0351	97.80	94.30	—	—
	{ Aerated	0.1094	0.0698	0.0396	99.00	98.00	—	—
10	{ Non-Aerated	0.1134	0.0817	0.0317	9.80	32.00	22.20	3.28
	{ Aerated	0.1094	0.0786	0.0308	9.90	33.20	23.30	3.35
1	{ Non-Aerated	0.1134	0.0939	0.0195	1.00	6.90	5.90	6.90
	{ Aerated	0.1094	0.0914	0.0180	1.00	8.40	7.40	8.40

6. *Effect of the conditions of cell reproduction on the final nitrogen coefficient of the yeast.* The following table shows that there is apparently some connection between the final nitrogen coefficient of the yeast and the conditions of cell reproduction, for the mean of the three quoted experiments seeded at 100 cells/*T* is 0.0026, at 10 cells/*T* is 0.0016, and at 1 cell/*T* is 0.0036.

Table VII.

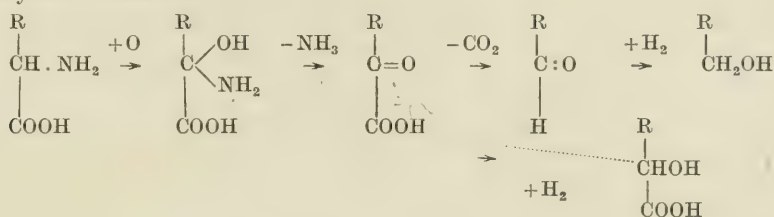
		Nitrogen coefficient of yeast $\times 10^4$		
		Initial	Final	
Experiments		<i>A</i>	<i>B</i>	<i>C</i>
Amino-acid	<i>A, B and C</i>	100 cells/ <i>T</i>	10 cells/ <i>T</i>	1 cell/ <i>T</i>
Leucine	21.7	25.0	14.0	38.0
„	18.0	24.0	16.0	35.0
Asparagine	18.0	30.0	17.0	35.0

PART II. CONSIDERATION OF THE THEORY OF THE MECHANISM OF THE REMOVAL OF NITROGEN FROM AMINO-ACIDS BY YEAST.

Fifteen years ago Ehrlich [1905] put forward his theory of the degradation of amino-acids by yeast, explaining the action as follows. The (NH_2) groups of the amino-acids are first split off from the parent molecule by the action of some desaminating enzyme, with the formation of the corresponding hydroxy-acid. The ammonia thus liberated is assimilated by the yeast and probably used in the construction of protein matter. At the same time the hydroxy-acid is subjected to what is presumably the ordinary zymatic action of the yeast, namely, the liberation of carbon dioxide and consequent formation of an alcohol, an action analogous to the supposed decomposition of lactic acid

under similar circumstances to carbon dioxide and ethyl alcohol. This theory states then that a hydroxy-acid is the intermediate product of the desamination.

Six years later, however, some work was published by Neubauer and Fromherz [1911] which carries Ehrlich's work a step further, in that they isolated a ketonic acid as the intermediate product of the action. They modify the theory of the action, for, in their opinion, the degradation is represented graphically as follows:



According to both these theories, the degradation of the amino-acid molecule is primarily caused by a hydrolysing enzyme, followed by a zymatic one, but Neubauer and Fromherz's explanation also requires the presence in the yeast of two others, an oxidising and a reducing enzyme.

The intermediate body, according to Ehrlich, is a hydroxy-acid, and Neubauer and Fromherz have succeeded in separating such a body, although they state it to be a reduction bye-product of the true intermediate body, the ketonic acid.

In any case the alcohol corresponding to the amino-acid, as shown in the above formulae, results: tyrosine, phenyl-amino-acetic acid, serine, and α -amino- α -methylbutyric acid, all have been shown by Ehrlich to yield the expected alcohol.

Hans Pringsheim [1906, 2] has also studied the desamination of the amino-acids more particularly in connection with the *Mucors*, and concludes from his results that for nitrogen to be assimilated from a complex molecule the group ($-\text{CO}-\text{CH}-\text{NH}$) must be present, and as proof of this gives the following list of bodies which he has succeeded in decomposing by moulds: glycine, alanine, leucine, tyrosine, aspartic acid, phenylglycine, allantoin, phenylalanine, and hippuric acid. He further states that a long side chain favours such a degradation.

This production of alcohols from amino-acids is, however, quite distinct from the ordinary zymatic action of the yeast, for both Ehrlich [1906] and Pringsheim [1906, 1] have proved that no amyl alcohol is formed from leucine by the action of active "acetone yeast" in the presence of sugar.

Thus the following conclusion naturally presents itself: There are two—in some ways different—actions taking place, (1) the desaminating action proper, and (2) the zymatic action. It appears probable that could these two actions be separated some light would be thrown on the extraction of nitrogen from amino-acids by yeast, and therefore it may be that by suppressing those

actions connected with the zymatic activity the presence in the yeast cell of a desaminating enzyme could be proved.

Effront [1908] apparently succeeded in doing this to a certain extent, for he found that in the decomposition of amino-acids by yeast in an alkaline medium he could demonstrate the presence of an enzyme, amidase, which under certain conditions liberates the whole of the nitrogen of amino-acids as ammonia, leaving various acids as the result of the action. For example, the whole of the amino-acid nitrogen of asparagine and part of the nitrogen of the yeast material itself are set free as ammonia in 96 hours. He also states that the enzyme can be obtained in solution.

Experiments carried out along the lines described by Effront showed two fertile sources of error, namely, bacterial growth and the presence in the liquid to be distilled of free alkali, both of which would nullify any results obtained [Effront, 1909].

The conditions of experiment are such that the yeast is dead, and a further proof that the action is quite independent of the life of the yeast was obtained by the use of dried, finely ground yeast instead of the pressed yeast before employed, the results showing that a little less than 75 % of the nitrogen was liberated as ammonia (Table IX, p. 469).

Thus the presence in the yeast cell of a desaminating enzyme, independent of the life of the yeast cell itself, seems to be proved, although it has been found impossible to extract the enzyme from the yeast in the manner described by Effront (*Experimental Section*, § 2).

Besides this desaminating ferment, amidase, however, another enzyme which causes hydrolysis analogous to the supposed hydrolysis by amidase was discovered in yeast by Shiga [1904]. This enzyme was arginase, and had previously been found in animal tissue by Dakin [1904] and its action more fully investigated by Kossel and Dakin [1904, 1, 2]. Its specific activity is the hydrolysis of arginine to ornithine and urea, although experiment proved the action to go further, the urea itself being hydrolysed.

Kossel and Dakin not only discovered this enzyme, but they also succeeded in separating it in a solid form from solution by precipitation [Kossel and Dakin, 1904, 3], and as the tissues which contain arginase act vigorously on asparagine, glutamic acid, etc., it seemed possible that this enzyme was the one at work in the experiments conducted by Effront. This possibility was more or less discountenanced by Effront's observation that amidase would not act in an acid medium, while the arginase of Kossel and Dakin is extracted by and is active in a 0.2 % acetic acid solution. Attempts at acid extraction, however, also proved abortive, and one is forced to the conclusion that the enzyme amidase is either in an insoluble state in its original form in the yeast tissue, or that it depends for its action upon a coferment which cannot be extracted by the means employed above.

If the action were a simple hydrolysis, malic acid should result from asparagine, but Effront states that the chief product of the action is propionic

acid. This being the case, the action must be a complex one and zymatic and reducing enzymes must also take part in the degradation. That yeast can, under suitable conditions, reduce the hydroxy-group of malic acid was proved by A. Fitz¹ who obtained calcium succinate from calcium malate. Reduction is also necessary in the theory of Neubauer and Fromherz.

Analysis of the liquid resulting from the action of yeast on asparagine under Effront's conditions revealed the presence of volatile fatty acids, apparently acetic and propionic, together with a non-volatile acid corresponding in molecular weight with malic which was present in an amount equal to about 60 % of the asparagine destroyed.

Presuming this removal of ammonia to be the first step in the fermentation of amino-acids, then malic acid—or ammonium malate—should be fermentable by yeast to yield ethyl alcohol alone. Experiment proved that under normal conditions of fermentation malic acid was unattacked by yeast, a result confirmed by some work of Oskar Emmerling [1899: reference to which was not found until after the work was completed] who found that while a pure culture of *B. lactis aerogenes* fermented malic acid to form succinic and formic acids with a little carbon dioxide and ethyl alcohol, pure cultures of brewery yeast had no action on malic acid, the apparent action of ordinary cultures being due to bacterial contamination.

It was thought, however, that the ammonium salt might act in an altogether different manner, for salts are often attacked while the acids themselves resist the action, *e.g.*, it has been stated that calcium malate is reduced by yeast to calcium succinate, and it seemed quite possible that the ammonium salt might serve as a source of nitrogen for the yeast cell. This was found to be the case, for, in the presence of sugar, yeast attacked ammonium malate, ethyl alcohol being the only product of the fermentation revealed by analysis of the resultant liquid. Effront states that the chief product of the action of amidase on asparagine is propionic acid, and consequently were the action of amidase the first step in the fermentation of this amino-acid then ammonium propionate should also be fermentable. This proved to be the case, although only to about half the extent of ammonium malate but quite sufficient to preclude the possibility of drawing any definite conclusions as to the rôle of malic acid in the fermentation of amino-acids.

Experimental.

1. *Desamination by yeast in alkaline solutions.* The method of conducting the desamination of amino-acids by amidase as given by Effront is as follows: 2 g. of asparagine and 10 g. of pressed yeast are mixed together with a little water, 6 cc. of *N* NaOH are then added and the mixture made up to 100 cc. with water. The ammonia formed as a result of the action of amidase on the

¹ Richter's *Organic Chemistry* (Smith), 1, 488. I cannot find reference to this work, the nearest being that of A. Fitz [1879] who found that certain schizomycetes acted upon malic acid to produce succinic and acetic acids.

asparagine is determined at intervals in a portion of the filtered liquid by distilling *in vacuo* with magnesia; controls of asparagine alone and of yeast alone are carried out concurrently.

The preliminary experiments were carried out in this manner, but as stated above it was found that abnormal results were obtained. Consequently in the following experiments toluene was in all cases added as an antiseptic and previous to distillation the sodium hydrate was neutralised by sulphuric acid¹. The following are typical results:

Table VIII.

Initial nitrogen in liquid	After 120 hours action		
	Total N	Ammoniacal N	% of initial N as NH ₃
1.1200	1.1295	0.8064	72
1.4500	1.1600	0.8477	74

2. *Attempts to obtain amidase in alkaline solution.* In a second parallel experiment the liquid was filtered after 72 hours and the filtrate divided into two portions; to the one 5 g. of asparagine were added, whilst the other was boiled to serve as a control. The liquids were kept at 37° for three days when the free ammonia was estimated, with the result that the control liquid was found to contain the same amount as the test itself, a result directly in opposition to the statement of Effront that under such conditions the enzyme is easily extracted.

3. *Attempts to obtain amidase in acid solution.* About 100 g. of pressed yeast were ground in a mill and extracted with 100 cc. of 0.2 % acetic acid for three days at 37°. The liquid was then filtered, and the filtrate, presumably containing the enzyme, divided into two parts and treated as in the alkaline extractions. The results were quite negative.

4. *Proof that the action of amidase is independent of the life of the cell.* An amount of dried yeast, finely ground, corresponding to the amount of pressed yeast used in the previous experiments was introduced into the amino-acid solution, and the action allowed to proceed as in the cases already described. The results obtained are given in Table IX.

¹ The form of distillation apparatus used was one devised by Brown [1903, 1] a modification of the Longi apparatus as used in the Sachsse-Schloesing-Longi method of ammonia estimation.

A great difficulty was at first experienced in these distillations by reason of the vigorous frothing which occurred when the pressure was reduced to about 50 mm. (at 40°) or when the temperature reached 35° if the pressure was previously reduced to 20 mm. It was found, however, that after raising the temperature to 40° and keeping the pressure at 100 mm. for about 15 to 20 minutes, the pressure could be diminished and the liquid would boil quite normally. The distillations were conducted at 40° and 12–15 mm. pressure. As a safeguard against sudden frothing carrying the liquid over into the receiver, a tap was inserted in the apparatus between the hot water jacket and the receiver so that on the slightest indication of frothing, the pressure in both the distillation flask and receiver could be immediately increased. It also served as a means of keeping the vacuum at the desired pressure in the first part of the distillation.

Table IX.

Time in hours	Total N in liquid	Total N as NH ₃ in liquid	% nitrogen as NH ₃
24	1.1199	0.3927	35.06
48	1.1200	0.7707	68.80
120	1.1205	0.8069	72.00

5. *Estimation of the volatile acids in resultant liquids.* The liquid, after filtration, was acidulated with sulphuric acid and distilled with additions of water as the liquid in the distilling flask became concentrated. The distillate was subsequently treated with an amount of baryta solution sufficient to neutralise the acid present, and the neutral liquid, after the separation of a certain amount of precipitate, evaporated to dryness in a weighed dish. The residue, after weighing, was treated with sulphuric acid and heated in order to remove the volatile acids. From the weight of sulphate obtained and the amount of barium salt decomposed the molecular weight of the volatile acid was calculated.

Table X.

Weight of barium salt taken	Weight of BaSO ₄	% Ba in salt	Mol. weight of acid if monobasic
1.7316	1.5448	52.50	64.40
1.0844	0.9581	51.90	65.80

The percentage of Ba in barium propionate is 48.5 and in the acetate 53.8; therefore the above results appear to suggest that the volatile portion of the products of the desamination of asparagine is a mixture of acetic and propionic acids.

6. *Estimation of the non-volatile acids in resultant liquid.* With regard to the non-volatile portion of the acid products it was found a matter of considerable difficulty to obtain a reliable method of estimation. Finally the following method of procedure was decided upon. The experimental liquid was neutralised with sulphuric acid heated to boiling point and treated with an excess of precipitated calcium carbonate, and after boiling for about seven minutes filtered. The clear filtrate was concentrated and filtered to remove any calcium succinate which separated out, and then treated with about ten times its volume of 95 % alcohol. This precipitated any calcium malate, which is practically insoluble in 85 % alcohol. It was found that a convenient method of procedure was to filter through a Gooch crucible, for the salt could then be easily taken into solution again if necessary, or transferred to an evaporating basin for decomposition with sulphuric acid.

The results given below tend to show that a considerable amount of malic acid is produced in the desamination of asparagine by yeast in the conditions of experiment described.

Table XI.

Weight of Ca salt taken	Weight of CaSO ₄	% Ca in salt	Mol. weight of acid
0.6166	0.4896	23.36	132.8
0.7392	0.5897	23.48	133.5

Calculated for calcium malate $C_4H_4O_5Ca$. Ca 23.25 %.

Molecular weight of malic acid = 134.

Total weight of calcium salt obtained from 2 g. asparagine = 1.3558 g.

Weight of asparagine corresponding to Ca salt obtained = 1.18 g.

Amount of asparagine decomposed (by nitrogen) = 1.623 g.

7. *Fermentation of malic acid and ammonium malate.* Investigation of the power of yeast to ferment malic acid was conducted on a solution containing 1 % malic acid and 10 % cane sugar with pure culture yeast, but the results showed that no acid was destroyed. In the case of the ammonium salt, the procedure was as follows: 1.0 g. malic acid was dissolved in water and the solution neutralised by ammonia; 20 g. of sugar and 20 g. of pressed yeast were added and the mixture made up to 250 cc. The solution was kept at 37° and a vigorous fermentation ensued which was practically over in three days. The progress of the action was followed in two ways (1) by estimation of the amount of nitrogen extracted from the liquid: (2) estimation of the decrease in the amount of malic acid present. Both series of determinations were carried out in the manner previously described, and yielded the results shown in Table XII which prove that ammonium malate is fermented by yeast.

Table XII.

Time in hours	(a) Nitrogen determinations			(b) Acid determinations		
	Nitrogen in liquid	Corresponding weight of salt	% salt fermented	Weight of Ca salt	Corresponding wt of (NH_4) salt	% salt fermented
0	0.1127	0.6762	Nil	0.7066	0.6902	Nil
24	0.0978	0.5868	13.20	0.6160	0.6017	12.82
96	0.0801	0.4806	28.90	0.5081	0.4964	28.08

8. *Products of the action of yeast on ammonium malate.* After the separation of the malic acid precipitate the filtrate was carefully evaporated to dryness at 40° *in vacuo*. This solution should contain ammonium carbonate (by the double decomposition of calcium carbonate and ammonium malate) and the calcium salt of any acid formed during the fermentation. The residue therefore of this solution was gently heated, with a result that practically the whole of it decomposed, proving it to be ammonium carbonate. The very slight amount of residue left undecomposed was quite inestimable.

Thus it appears that the only product of the fermentation of ammonium malate is ethyl alcohol.

9. *Fermentation of ammonium propionate.* The results of experiments are given in Table XIII, which show that this salt is also decomposed by yeast. The products of the action were not analysed.

Table XIII.

Time in hours	(a) Nitrogen determinations			(b) Acid determinations		
	Nitrogen in liquid	Corresponding weight of salt	% salt fermented	Weight of Ba salt	Corresponding wt of (NH_4) salt	% salt fermented
0	0.0916	0.5954	Nil	0.9350	0.6013	Nil
24	0.0875	0.5685	4.52	0.8868	0.5703	5.16
96	0.0786	0.5110	14.14	0.7980	0.5132	14.65

PART III. INVESTIGATION OF THE EFFECT OF VARIATION IN THE AMOUNTS OF AMINO-ACID AND OF SUGAR ON THE ASSIMILATION OF NITROGEN FROM AMINO-ACIDS BY YEAST.

It has already been found (Part I) that the amount of nitrogen extracted from an amino-acid by yeast varies very considerably with the change in the concentration of the yeast cells, but, so far, the experiments have always been made with an excess of amino-acid and a large amount of sugar available for fermentation.

In the first place zymatic activity is necessary for the absorption of nitrogen by yeast, the results of a series of experiments conducted by treating amino-acid solutions with yeast in the absence of sugar proving that no nitrogen was extracted. On the other hand a further series, in which the relative rates of fermentation of sugar and decomposition of amino-acids were determined, showed that, although the one is no definite function of the other, yet the activity of the zymase does exert a stimulating effect on the desamination of the amino-acids.

A rapid fermentation, however, does not mean rapid absorption of nitrogen, for experiment shows that if fermentation is extremely rapid and consequently soon over, the desaminating enzymes apparently have not sufficient time to reach a state of activity and as a result little absorption of nitrogen takes place.

As regards the effect of the second factor mentioned above, viz., variation in the amount of amino-acid, investigation shows, as would be expected, that the quantity of nitrogen extracted is not proportional to that of amino-acid originally present, although the results obtained give some evidence that the nitrogen-content of the solution does influence the nitrogen absorption.

Worthy of note are the varying amounts of nitrogen which a yeast will assimilate under conditions apparently similar. Thus it has been observed that, with a 10 % sugar solution containing 1 % nitrogen as asparagine and seeded at the rate of 100 cells/*T*, the nitrogen absorbed in 24 hours has varied from 21.7 % up to 37.2 % of the amount originally present. It has, indeed, been already shown that the nitrogen content of the seed-yeast has an influence on the absorbing capacity, but in a number of cases two samples of the same yeast, after apparently identical treatment, have absorbed appreciably different amounts of nitrogen. It seems probable that such differences depend on physiological conditions of which we are as yet ignorant.

It has been found from the large number of fermentations conducted in this investigation that on an average a normal yeast seeded at the rate of 100 cells/*T* will extract in 24 hours 0.0250 g. of nitrogen per 100 cc. of the fermenting solution, but after the lapse of 48 hours—when the extraction will generally have reached its maximum—this amount may have increased to 0.0450 g.

Experimental.

1. *Action of living yeast on amino-acids in the absence of fermentative activity.* A washed brewery yeast was seeded into a solution of asparagine containing 0.2 % of nitrogen (1.072 g. of asparagine per 100 cc.). The liquid was subsequently maintained at 37° and at intervals of 12 hours quantities of about 75 cc. were withdrawn and filtered, the nitrogen being estimated in 50 cc. of the clear filtrate. (Schleicher and Schüll's No. 572½ filter papers are found to be especially suitable for the complete separation of yeast cells from fermented solutions.) The results thus obtained are as follows:

Table XIV.

Time in hours	0	12	24	36	48	72
Nitrogen in liquid (g. per 100 cc.)				0.2016	0.2002	0.2022	0.2038	0.2040	0.2044

The only conclusion to which these figures lead is that without fermentation no assimilation of nitrogen takes place. No importance can be attached to the apparent slight decrease of nitrogen during the first 12 hours, although later experiments seem to show that, if the yeast is in a healthy condition, assimilation may occur to a very slight extent, this probably being due to the still incomplete subsidence of the stimulus caused by a previous fermentation.

The gradual increase observed in the nitrogen-content of the solution after the initial 12 hours is of greater importance than is at first evident; this point is considered in detail in Part IV of the present paper.

2. *Relationship between nitrogen assimilation and zymatic activity.* The relative rates of assimilation of nitrogen and of fermentation were determined by means similar to those employed in the experiment described above, except that the solution here contained 10 % of cane sugar. The progress of the fermentation was followed by estimations of the sugar content of the liquid according to Bertrand's method. The results obtained are shown in Table XV.

Table XV.

Time in hours	0	6	12	24	30	36	48	60
% sugar in solution	100.00	71.00	31.00	5.00	2.75	1.20	1.20	1.20
% nitrogen in solution	100.00	—	78.00	62.80	—	49.70	42.40	42.50

Examination of these figures reveals two important points: (1) the rate at which nitrogen was assimilated in this experiment was practically constant for the first 30 hours, but subsequently underwent rapid diminution until after 50 hours absorption of nitrogen had practically ceased: (2) although fermentation of the sugar had practically ceased after 30 hours, the assimilation of nitrogen continued for 50 hours.

3. *Effect on nitrogen assimilation of rapid fermentation.* Very rapid fermentation was induced by seeding three 10 % sugar solutions containing 1 % nitrogen as asparagine with yeast at the rate of 300 (*A*), 200 (*B*), and 100 (*C*) cells/*T* respectively. After the lapse of 12 hours visible fermentation had

ceased in *A* and had nearly ceased in *B*, whilst *C* was still fermenting vigorously. At the end of 24 hours, estimations of nitrogen in the usual way gave the following results:

Table XVI.

Experiment	<i>A</i>	<i>B</i>	<i>C</i>
Rate of seeding	300	200	100
% nitrogen assimilated	8.50	16.80	22.60

4. *Effect on nitrogen assimilation of the amount of available nitrogen present.* The liquids employed consisted of 10 % solutions of cane sugar and a saturated solution of asparagine, these being mixed in proportions to bring the nitrogen content to 2.0, 1.0, 0.4 and 0.2 % respectively. These mixed solutions were then seeded with yeast at the rate of 100 cells/*T* and allowed to ferment at 37°. The fermentations were apparently complete in 24 hours, at the end of which time the amounts of nitrogen remaining in the various solutions were estimated.

The following are the results obtained:

Table XVII.

Experiment	Total nitrogen as g. per 100 cc.		% nitrogen assimilated
	Initial	Final	
<i>A</i>	2.0000	0.9000	55.00
<i>B</i>	1.0000	0.5083	49.17
<i>C</i>	0.4000	0.2889	27.78
<i>D</i>	0.2000	0.1549	22.55

Expressed as ratio, these figures are as follows:

Total nitrogen introduced in *A* : *B* : *C* : *D*; 100 : 50 : 20 : 10.

Total nitrogen assimilated in *A* : *B* : *C* : *D*; 100 : 44.7 : 10.1 : 4.1.

It must therefore be concluded that the smaller the amount of nitrogen available for extraction from the solution, the less is the power of the yeast to extract the nitrogen.

Further elucidation of this point was attempted by means of a series of experiments in which a 10 % solution of cane sugar containing 0.03, 0.04, and 0.05 g. respectively of nitrogen as leucine per 100 cc. of solution was fermented in the manner described above. Leucine was chosen here because asparagine contains both "amino" and "amido" nitrogen, and previous experiments had shown that nitrogen is not absorbed at the same rates from these two groups. It was therefore thought that with such small amounts of nitrogen present in the solutions here involved, the use of leucine would present fewer difficulties than that of asparagine. Estimations of the amounts of nitrogen present in the solutions were made at intervals of 24 hours, with the following results:

Table XVIII.

Experiment	Initial nitrogen	Final nitrogen			
		24 hours	48 hours	72 hours	96 hours
<i>A</i>	0.0300	0.0120	0.0030	0.0037	0.0050
<i>B</i>	0.0400	0.0170	0.0060	0.0045	0.0049
<i>C</i>	0.0500	0.0230	0.0107	0.0050	0.0045

These numbers confirm that (1) the greater the amount of available nitrogen the greater the initial rate of extraction, and (2) that, in all cases but *C*, before all the nitrogen has been absorbed, an increase in the nitrogen content of the solution is to be observed. This latter is a most important point as is shown in the final part of this paper.

PART IV. THE APPARENT EXCRETION OF NITROGEN BY YEAST.

Towards the end of Part III of this communication it was pointed out that experiments seemed to indicate that under certain circumstances yeast may lose nitrogenous matter, and, for want of a better term, this may be called "nitrogen excretion."

A preliminary experiment showed that a yeast of high nitrogen content (in the case in point the yeast contained 10.3 % of nitrogen on the dry matter) would, under certain conditions, lose about 35 % of its nitrogen in fermenting a pure cane sugar solution. That this excretion did not wholly consist of bodies detrimental or useless to the yeast cell was shown by the fact that these bodies could be partly reassimilated by another yeast suggesting that the action is not a true excretion. In the following pages, however, this term has been applied to this peculiar action of the yeast.

Apparently the increase in the nitrogen content of a fermenting solution, resulting from this action, may be explained in two ways. It may be the result of (1) a "normal" excretion of nitrogen by the yeast cell, or (2) a disintegration of the cells consequent upon the commencement of auto-digestion. If the action is normal then it would be thought that a high-nitrogen yeast¹ could, cell for cell, excrete more nitrogen than a starved yeast. On the other hand, if the excretion is a pathological condition, then the starved yeast should be more prone to auto-digest and consequently should release its nitrogen constituents more rapidly. Experiment showed, however, that this was not the case, but that conversely a well-nourished, high nitrogen yeast excreted more nitrogen in fermenting a certain amount of sugar than a starved yeast. Due however to the differences in the fermentative powers of yeasts under certain conditions difficulties in comparison were found as is explained in the experimental section.

The question naturally arises, "Is this action contingent on the life of the yeast cell, or only connected with the zymatic activity?" Experiment soon proved that the action was essentially a living one, for a sample of dried yeast containing very active zymase was introduced into a sugar solution in amount to make it comparable with the living yeast experiments, and it was found that between the sixth and one hundred and twentieth hours of fermentation, the liquid only gained about one-eighth the amount of nitrogen excreted by the living yeast. Another experiment in which all the enzymes had been de-

¹ It must be emphasised that in all these experiments one race of yeast was used; reference to a high-nitrogen yeast always means a well-fed yeast, containing a large percentage of nitrogen.

stroyed gave a similar result, proving that the action is essentially a vital phenomenon.

This being the case, one would naturally expect the amount of excreted nitrogen to be proportional to the number of cells present, and experiments seeded with 10, 20, 30, etc., to 150 cells per *T*, showed that the yeast yielded to the liquid while fermenting a 10 % cane sugar solution amounts of nitrogen proportional to the number of cells present. The initial nitrogen coefficient of the yeast was 0.0019, and the average loss per unit cell was 0.00011 in two days, and 0.00014 in five days, that is to say, the yeast excreted 5.78 % of its nitrogen in 48 hours, and 7.36 % in 120 hours.

Not only, however, is this activity dependent upon the life of the yeast, but it is also dependent upon the zymatic activity. For example, fresh yeast seeded at 100 cells/*T* into pure water yielded 0.41 g. N per 100 cc. in 72 hours, which amount had not perceptibly increased after 120 hours, while a similar seeding into a 10 % cane sugar solution gave 0.71 g. in 72 hours and 1.70 g. in 120 hours. There is, therefore, some connection between nitrogen excretion and fermentative activity. Before studying this connection it was necessary to consider to what extent the time factor enters into the excretory action.

A solution of invert sugar was seeded with yeast at the rate of 75 cells/*T* and maintained at 20° during fermentation. At intervals of time the progress of the fermentation was estimated, and at the same time the amount of nitrogen in the solution was determined. From confirmatory series of experiments it was found that the rate of fermentation of the invert sugar present was constant for about 16 to 20 hours, then rapidly decreased, until at 36 hours it had ceased. On the other hand the nitrogen excretion progressed regularly for about 75 hours, after which it rapidly decreased, and after the 110th hour the action had apparently terminated.

The constant rate of excretion was approximately, in two series *A* and *B*, 0.000419 and 0.000384 g. N per hour for 75 cells/*T*. Compared with the results of a previous experiment the excretion in 24 hours per unit cell would be in these two cases 0.000134 and 0.000123 as against 0.00011 previously found. Variations to this extent have constantly been found to occur, and are probably due to the exact physiological state of the initial seed yeast.

The most important conclusion to be drawn from these two experiments is that although there is no direct relation between fermentation and excretion, the activity of the zymase stimulates the latter action, and the cessation of the metabolic changes thus stimulated does not coincide with the apparent termination of the fermentative activity. As would be expected the zymatic and excretory powers of the yeast cell are two distinct functions, but the activity of the former in some way stimulates the latter into action.

This stimulatory action of fermentation was proved very clearly as follows. Water was seeded with fresh yeast at the rate of 100 cells/*T*, and allowed to stand for 30 hours during which time two nitrogen determinations were made. At the thirtieth hour sugar was added and nitrogen determinations

were again made on the liquid at subsequent intervals of time. No sugar was added to a second experiment and the differences in the amounts of nitrogen found in the two cases were very striking. For example after 72 hours the amount of N in the sugar solution was 1.07 g. per 100 cc. and 0.41 g. in the aqueous suspension.

Consequently nitrogen excretion is, to a certain extent, dependent on the zymatic activity of the yeast, and as the expression of the latter is the amount of fermentation, it appeared desirable to investigate the effect of the variation of the amount of sugar available. Solutions containing differing amounts of cane sugar were fermented with samples of the same yeast, and nitrogen determinations made after 24, 48, 72, and 120 hours. The results obtained show the great stimulating effect on the nitrogen excretion of increase of the amount of available sugar from 1 % to 5 %—confirmatory results being obtained for all periods of time. The stimulatory effect rapidly decreases, and above 10 % sugar increase does not result in an appreciable increased rate of nitrogen excretion.

The nitrogen thus excreted may be derived from

(1) the reserve nitrogenous food material in the yeast cell, in which case the loss of nitrogen should terminate with the exhaustion of that reserve material,

(2) the nitrogenous tissue of the cell itself and, if this is the case, it is possible that the continued action may result in the death of the organism.

It is, of course, difficult to keep a yeast in a state of prolonged fermentative activity in a solution because of the accumulation of the products of fermentation which eventually destroy the zymatic activity, but a similar state was obtained by continually transferring a yeast from one sugar solution to another as soon as fermentation finished in the first case. In this way it was found that after five such fermentations the zymatic activity of the yeast was practically destroyed, although, on introducing the exhausted yeast into a wort, after 24 hours 85 % of the cells appeared to be in good condition and 55 % of them were budding. Consequently after the fifth fermentation although exhausted the cells were not dead, and rapidly regained a normal condition. During these successive fermentations the yeast lost 37.8 % of its nitrogen, its nitrogen coefficient decreasing from 0.00200 to 0.00124. This experiment demonstrates that a yeast can lose more than a third of its nitrogen, but that further loss results in the disablement of the cell, with a consequent loss of its fermentative capacity.

All the work so far described may be said to have been concerned with yeast under abnormal conditions, the abnormality consisting in the absence of nutrient matter from the sugar solution. Much work of a fruitless character was undertaken to determine whether this excretory action takes place while the assimilatory processes are also progressing, such, *e.g.* as the tracing during fermentation of solutions containing small amounts of asparagine, leucine, alanine, etc., of the amino-index, for the index of the excreted nitrogenous

matter is not that of the amino-acid. Finally it was found that the best method was precipitation of the fermented solution by phosphotungstic acid, for 35–37 % of the excreted matter is precipitated by this reagent. The method employed consisted of the fermentation of sugar solutions containing non-precipitable nitrogenous matter, with subsequent estimation at intervals of the nitrogen precipitated by the reagent.

The results conclusively proved that even when rapid assimilation of nitrogen was taking place, excreted nitrogen could also be found in the solution, although the absolute amount under different conditions was subject to wide variation, the cause of which was not determined.

Experimental.

1. *Preliminary experiment.* A 20 % cane sugar solution was seeded with yeast containing a high percentage of nitrogen, at the rate of 100/*T*, and during fermentation carried out at 10° (in order to obtain a slower action) the nitrogen in solution was determined every 24 hours. The yeast employed was a sample of brewer's yeast and from Table XIX it will be seen that during its fermentation (which, judged by the settlement of the yeast, lasted about seven days) it lost a very considerable amount of nitrogen to the liquid.

Table XIX *a.*

Time in hours	24	48	72	96	120	240
N in liquid as g. per 100 cc.			0.0760	0.0840	0.1520	—	0.2440	0.3360

Table XIX *b.*

	Total N in solution	Total N in yeast	% N in yeast on dry matter	% N lost by yeast
Initial	Nil	1.3520	10.30	—
Final	0.3360	1.0160	7.74	24.85

2. *Assimilation by yeast of nitrogenous matter previously excreted.* After the fifth day of fermentation, 250 cc. of the solution was extracted, filtered and vacuum distilled at 40°. In this way the residue was obtained free from alcohol and water at a temperature at which practically no change would have taken place in the constitution of the amino-acids. The residue so obtained was dissolved in 10 % sugar solution and fermented with a normal yeast in the ordinary manner. The yeast contained 8.2 % nitrogen on the dry matter; nitrogen determinations made on the solution gave the following results:

Table XX.

Time in hours	0	48	120
Total N in solution	0.0620	0.0240	0.0370

The fermentation was practically over at the end of the second day when 61.30 % of the original nitrogen had been reassimilated by the yeast, and it is interesting to note that at the end of five days a further excretion had taken

place. This suggests that about one-third of the original excretion is not assimilable by the yeast, which may be that portion precipitable by phosphotungstic acid.

3. *Comparison of the excretory powers of yeast of high and low nitrogen coefficient.* Sample *A* was a well-nourished yeast containing 9.2 % nitrogen on the dry matter, while *B* only contained 7.0 % of nitrogen. The yeast was introduced at the rate of 100 cells/*T* into 10 % sugar solution and fermentation allowed to proceed at 37° for four days, during which time determinations of the nitrogen content of the solution were made after 48 hours and at the termination of the experiment.

Table XXI.

Yeast condition	% N in yeast on dry matter	N in liquid	
		48 hours	96 hours
High nitrogen	9.2	0.3052	0.3648
Low nitrogen	7.0	0.2027	0.3500

The starved yeast was sluggish of action and did not finish fermentation until the expiration of four days, the well-fed yeast had completely finished in less than 48 hours. The figures show the stimulatory effect of fermentation on the excretion of nitrogen, an effect which is more fully studied in a later experiment.

4. *Comparison of living and air-dried yeast as regards nitrogen excretion.* A yeast which had been air dried at 37°, and afterwards ground up in a mortar, was used, the preparation having been made some 12 months before use. Sufficient was added to a 10 % sugar solution to bring the nitrogen content of the solution up to 0.1352 N per 100 cc., thus making comparison with the experiment previously described possible. The mixture was allowed to stand at 37° and a vigorous fermentation took place, showing that the zymase was still in a state of activity. After periods of 6 and 120 hours the nitrogen content of the solution was measured, the amount of nitrogen introduced into the solution mechanically being obtained from the first reading.

Table XXII.

Experiment	N in solution g. per litre	
	6 hours	120 hours
Dried yeast	0.0304	0.0568
Living yeast	0.0150	0.2440

In the case of the dried yeast it is obvious that 0.0304 g., or nearly 60 % of the total amount of nitrogen given to the solution, was introduced mechanically in a soluble form, while it is to be noted that in 120 hours the *total* amount given to the liquid is less than 25 % of that excreted by the living cell.

5. *Comparison of living yeast with one in which all the enzymes had been destroyed.* The experiment was conducted on exactly parallel lines, except that yeast previously dried at 95° was used, and gave the results set out in the following table, in which the figures for living yeast are shown for purposes of comparison.

Table XXIII.

Experiment	N in solution g. per litre	
	6 hours	120 hours
Dried at 95°	0.0535	0.0862
Living yeast	0.0150	0.2440

6. *Effect of varying the amount of yeast present.* A series of experiments was made in which 10 % sugar solution was seeded with yeast at the rate of 10, 20, 40, 80, 150, and 320 cells/*T*. On plotting the results it became apparent that as far as this series went, the excretion varied in a regular manner with the amount of yeast present. The latter points, however, were so far apart in comparison with the first three, that the experiments were repeated in an extended form, 15 fermentations being carried out, the seeding being from 10 to 150 cells per unit volume. Nitrogen determinations were made at the end of 48 and 120 hours, those in the lower seedings being made on amounts of liquid varying up to 250 cc. The results—Table XXIV—are given as grams of nitrogen per litre.

Table XXIV.

Rate of seeding ...	10	20	30	40	50	60	70	80
Nitrogen in solution after 48 hours ...	—	0.021	—	0.048	0.054	0.064	0.076	0.122
Nitrogen in solution after 120 hours ...	0.025	0.024	0.050	0.055	0.068	0.081	0.104	0.110
Rate of seeding ...	90	100	110	120	130	140	150	
Nitrogen in solution after 48 hours ...	—	—	0.116	0.132	0.136	—	0.144	
Nitrogen in solution after 120 hours ...	0.131	0.129	0.144	0.172	0.208	0.192	0.221	

The results are expressed graphically in Fig. 1, and it must be remembered on examining these figures that the vertical scale is a very large one, so that, excluding the 80 cell experiment in the second day, the greatest error occurs in the 120 cell experiment, and is equal to 0.00084 g. N per 100 cc.

The curves show without doubt that the amount of nitrogen excreted is proportional to the amount of yeast introduced into the sugar solution, other factors being constant.

The following table shows, in brief, the loss of nitrogen by the yeast.

Table XXV.

Time	Nitrogen coefficient $\times 10^4$		% N lost by yeast
	Initial	Final	
48 hours	19.0	17.9	5.78
120 hours	19.0	17.6	7.36

7. *Effect of the time factor in nitrogen excretion.* A solution of invert sugar containing 10 g. per 100 cc. was seeded with yeast at the rate of 75 cells/*T* and allowed to ferment at 20°. For the first two days the amount of sugar fermented and the nitrogen excreted were determined every two hours from

8 a.m. to 8 p.m. and afterwards with varying intervals of time for five days¹. The following are the results of two experiments:

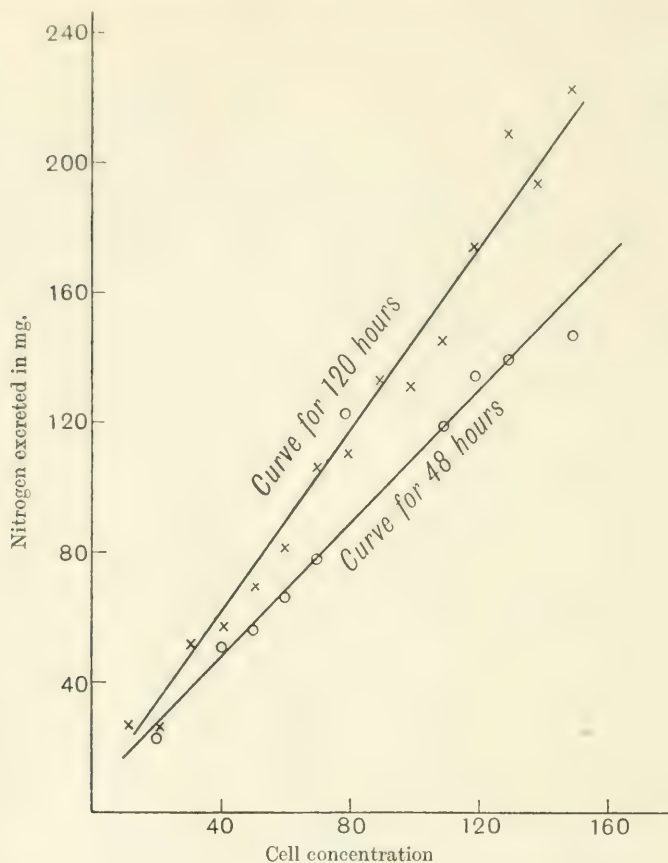


Fig. 1.

Table XXVI.

Experiment A.

Time in hours	2	4	6	8	10	12	24	26	28	30	32	34
Sugar fermented	1.70	1.80	2.90	4.60	5.50	6.90	8.70	9.50	9.62	9.73	9.85	9.87
N excreted*	—	0.90	0.74	0.76	1.26	0.84	1.18	—	1.28	—	1.34	1.30
Time in hours	36	48	52	56	60	72	78	84	96	102	120	132
Sugar fermented	9.88	9.90Constant.....									
N excreted*	1.56	1.66	1.70	1.64	1.94	2.14	2.12	2.12	2.02	2.20	2.22	2.26

* As g. $\times 10^2$ per 100 cc.

¹ The invert was prepared from cane sugar by taking a 40 % solution and hydrolysing in a steam steriliser for three hours with 5 cc. of strong hydrochloric acid per litre of solution. The optical rotation was then found to be constant, the acid was neutralised with sodium hydrate, and the solution diluted until it contained 10 % invert sugar. The determinations of the invert were made according to the method of Bertrand (*Manipulations de Chimie Biologique*).

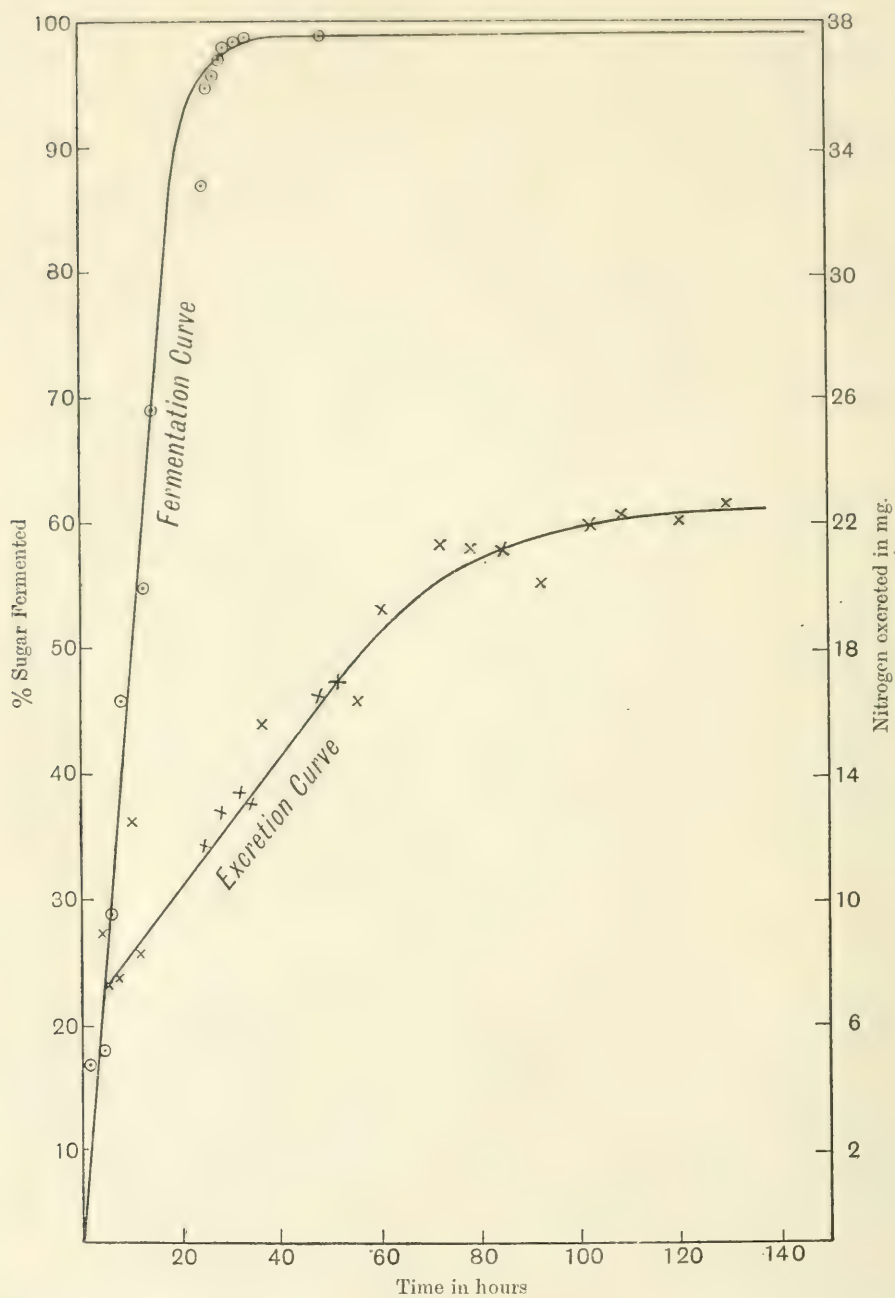


Fig. 2

These figures have already been studied in the theoretical part of the paper, and it only remains to be said that graphically they demonstrate very clearly the conclusions drawn.

10. *Effect of exhausting the fermentative powers of the yeast.* A litre of 20 % cane sugar solution was seeded with yeast—nitrogen coefficient 0.0020—at the rate of 100 cells/*T*, and the fermentation allowed to proceed at 37°. At the conclusion of the fermentation—about 32 hours—the yeast was washed once

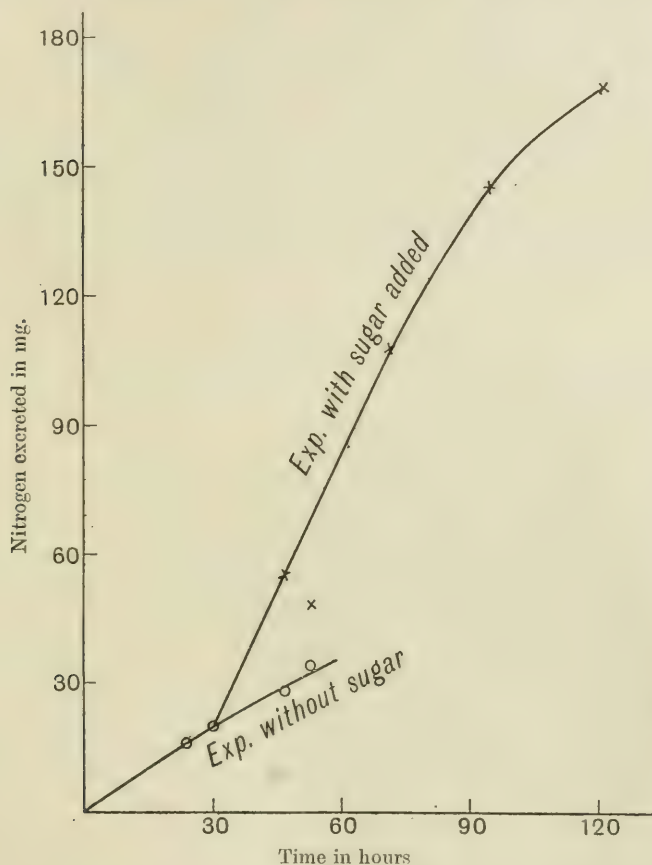


Fig. 3.

by decantation and filtered off through a Buchner funnel. The liquid, which was not quite clear, was treated with alumina cream, refiltered, and the perfectly bright filtrate acidulated with 100 cc. *N*/20 sulphuric acid (to prevent the escape of any ammonia) and concentrated to 200 cc. The nitrogen was determined in an aliquot portion of this solution.

The previously separated yeast was introduced into a further litre of 20 % sugar solution and allowed to ferment at 37°. The process of separation was carried out as described above, at the termination of the fermentation and the

whole process repeated. After the third fermentation the yeast had assumed an appearance altogether different from its original condition, for, although the cells looked perfectly healthy and approximately 5 % of them were budding, the mass was of a slimy, sticky nature, extremely difficult to filter. This appearance was much more marked after the fourth fermentation, which had been of a sluggish nature, and had taken much longer to commence, and when the yeast was introduced into the sugar solution for the fifth time the action was extremely slow. The yeast separated from this fermentation had apparently lost its zymatic power, but on being introduced into a wort (gravity 1053) it was found that after 24 hours about 85 % of the cells seemed to be in a fairly good condition and about 55 % of them were budding.

The revived yeast was again sown into wort, and after a further 24 hours only a very few poor cells were to be seen, and the majority were budding, thus demonstrating that although after the fifth fermentation the yeast was very exhausted, the cells were not dead. The following figures show the amounts of nitrogen lost during the successive fermentations:

Table XXX.

No. of fermentation	1	2	3	4	5
N excreted during fermentation			0.1160	0.1554	0.1967	0.1596	0.1293
Total amount of N excreted					0.7570		
Total N originally in the yeast					2.0000 g.		
% N lost during five fermentations					37.85		

There is apparently no explanation of the irregularity of the amounts of nitrogen excreted during different intervals, unless it be, and this is perhaps an important factor, that the aeration of the cells between the successive fermentations varied in amount and in efficiency; this might easily result from the varying states of the yeast and consequently the slightly different methods of filtration employed. However, the experiment demonstrates that during fermentation the yeast has excreted 37.8 % of its nitrogen, and not only this, but that further loss results in the disablement of the cell, with a consequent loss of its fermentative capacity.

11. *Determination of excretory matter in the presence of amino-acids.* Leucine was added in varying amounts to 20 % cane sugar solutions and fermented with yeast introduced at the rates of 50 and 100 cells/T; controls were made in which no yeast was added in one case and no sugar in another. At intervals the nitrogen was estimated as usual, and at the same time 50 cc. of the clear filtrate treated with 5 cc. phosphotungstic acid solution (10 % phosphotungstic acid in 5 % sulphuric acid). The mixture was stirred and the precipitate which formed allowed to settle, which it did very well in about 12 hours. The supernatant liquid was then decanted off and the precipitate—which in character was of a rather heavy, flocculent nature, and of an almost white or pale clay colour—mixed with about 15–20 cc. of the precipitant [Brown, 1903,

2] and filtered through an ordinary filter paper of a fairly loose texture¹; nitrogen determinations were subsequently made on the clear filtrate. Table XXXI shows the figures obtained for one such series.

Table XXXI.

Exp.	% sugar	Initial N as amino-acid	Final N after 48 hours	N pptd by phospho- tungstic acid	Change in N content of solution
<i>A</i>	0	0	0.0052	0.0020	+0.0052
<i>B</i>	0	0.0584	0.0600	Trace	+0.0016
<i>C</i>	20	0	0.0528	0.0200	+0.0528
<i>D</i>	20	0.0584	0.0308	0.0232	-0.0276
<i>E</i>	20	0.0630	0.0500	0.0232	-0.0130
<i>F</i>	20	0.1168	0.0552	0.0306	-0.0616

These figures show that:

1. Where fermentation has not occurred, no precipitable nitrogen, or practically none, appears in the solution.
2. The greater the amount of nitrogen assimilated, the greater the amount of precipitable nitrogen found.

GENERAL CONCLUSIONS.

1. *Factors which influence the assimilation of nitrogen by yeast.*

- (a) Excess of yeast ensures the removal of the greatest *total* amount of nitrogen.
- (b) During active fermentation the greater the coefficient of multiplication, the greater the amount of nitrogen assimilated by *each* cell.
- (c) Active reproduction may result in a lowering of the nitrogen coefficient, even when a large amount of available nitrogen is present.
- (d) The final nitrogen coefficient of a yeast is independent of the initial coefficient, and tends to reach a value constant for any particular conditions of reproduction.

2. *Mechanism of the extraction of nitrogen from amino-acids by yeast.*

- (a) The amidase of Effront does not (as stated by him) produce solely a volatile acid by its action on asparagine, but also a non-volatile acid, probably malic acid.
- (b) If malic acid represents the first stage in the degradation of asparagine, it is not fermented as such, for the acid is unattacked by yeast. It may combine with NH_3 to form the ammonium salt which is completely destroyed by fermenting yeast, ethyl alcohol being the only recognised product of the action.

¹ The first runnings had always to be returned, but after a few minutes the filtrate came through quite bright. The clear liquid was warmed to about 40° and treated with ground baryta with constant stirring, in order to remove the excess of phosphotungstic and sulphuric acids. A slight excess only of the baryta was added, and the liquid allowed to settle, the clear liquid decanted off, and the remainder filtered and the precipitate washed. The excess of baryta was then removed by carbon dioxide, and the nitrogen estimated in the filtrate. It will be understood that in face of the number of processes extreme care was required to obtain concordant nitrogen readings.

(c) Propionic acid (which Effront states results from the action of amidase on asparagine) is not fermentable, and its ammonium salt is attacked with difficulty by yeast.

3. *The influence of the available amino-acids and sugar on the nitrogen assimilation by yeast.*

(a) Fermentative activity is essential to nitrogen assimilation. The two actions are not proportionate, but the former stimulates the latter, and, once induced, desamination may continue after zymatic activity ceases.

(b) Excessive zymatic activity does not ensure rapid nitrogen assimilation, in fact a quick fermentation results in only a small amount of nitrogen being extracted.

4. *Apparent excretion of nitrogen by yeast.*

(a) During fermentation yeast continually loses nitrogen to the liquid, and this action has been called "nitrogen excretion." Cases have been noted where more than 33 % of the yeast nitrogen was so lost, but further loss resulted in the disablement of the cell.

(b) Nitrogen excretion is dependent on the *life* of the cell, and takes place even when nitrogen is being assimilated.

(c) The bodies so excreted can be used by yeast as a source of nitrogen under suitable conditions.

(d) Increase in the amount of sugar available for fermentation increases the rate of excretion, especially between the limits 1-5 % sugar, and although zymatic activity is necessary for nitrogen excretion, the two are not proportionate, nor does the cessation of the former result in an immediate cessation of the latter.

The Author desires to acknowledge his great indebtedness to the late Professor Adrian J. Brown, F.R.S., for the kindly advice and sympathy extended to him during the course of the above research (which was concluded prior to 1914); to Mr T. H. Pope, B.Sc., for his keen interest in the work, and to Professor A. Harden, F.R.S., for his great assistance in publication.

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XLI. THE RELATIONSHIP OF LECITHIN TO THE GROWTH CYCLE IN CRUSTACEA.

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THE period leading up to moulting in Crustacea is characterised by certain metabolic and anatomical changes, some of which we have dealt with in previous papers [Paul, 1914; Paul and Sharpe, 1916]. Briefly, these are as follows. There is a marked increase of fat, glycogen, and calcium in the liver, the last reaching as much as 20 % of the dried weight of the organ. At the same time mitosis occurs rapidly in the cells of the integument and other tissues of the body, but no increase in the size of the animal takes place till the rigid shell is cast and rapid absorption of water makes the animal increase in size.

When the moult is complete, an exodus of stored material takes place from the liver; the fat is broken down to glycerol and lower fatty acids before it enters the blood, and calcium is carried off as soaps of lower fatty acids to be deposited in the integument as calcium carbonate. Cell proliferation in the body then ceases, but increase in the size of the cells is marked.

After a period of rest the crab again stores material in the liver in preparation for the next moult. It has been found that as the period of moulting approaches the liver increases in weight relatively to the body. We have therefore employed the term *hepatic factor* to denote this relationship, and have applied it to the figure obtained by dividing body weight by weight of fresh liver. Shortly after moulting this factor may be 10 or 12, but as the storage goes on it may fall to five or even lower [Paul, 1915]. We therefore use the hepatic factor to indicate proximity to moulting.

Fat is present in the liver in quantities varying from 20 to 30 % of the total gland. In this fat we found phospholipins. The question arose as to the relationship of these substances to the metabolic cycle, and we have examined many individuals of the Decapod Crustacea to determine what it is.

The results of four of these examinations carried out on crabs (*Cancer pagurus*) at different periods between the moults are to be found in the table and graph hereunder.

METHODS.

The legs were removed by the production of autotomy [Paul, 1915] at the breaking-planes, and the weight of the body was then taken. The fresh liver was removed and put into ether-alcohol mixture (50 %). The fats were extracted by repeated washings with ether and hot alcohol and weighed. The fat was then ashed and the total amount of phosphorus present was determined by the ordinary molybdate method. Using the formula $C_{43}H_{85}O_8NPH$ [MacLean, 1918] this phosphorus was calculated to lecithin.

We consider the above method more exact than that in which acetone is employed to precipitate the phospholipins as such, and the calculation has been made to *true lecithin* [MacLean, 1918] in all cases, for purposes of comparison only.

RESULTS.

We find that the amount of fat in the liver increases both in total quantity and relatively to the weight of the body and liver as the animal approaches the moult.

It is also made clear that the amount of phospholipin in the liver (calculated to lecithin) diminishes both in total quantity and relatively to the weight of the body and liver as the animal approaches the moult.

CONCLUSIONS.

The relationship of phospholipin metabolism to developmental changes has been the subject of varied researches.

Noël Paton has shown that lecithin accumulates in the ovary of the salmon at the expense of the inorganic phosphorus of the muscle [1898]. The same author has indicated the increased percentage of lecithin in liver fats of mammals during starvation, and has associated it with the storage of phosphorus for nuclein formation [1896]. Hoppe-Seyler has demonstrated the high lecithin content of embryonic tissues [1887] and Plimmer and Scott have indicated the marked diminution of lecithin in hens' eggs during development of the embryo [1909]. More recently Brailsford Robertson, studying the development of certain sea-urchin eggs, has shown that there is a marked decrease of lecithin whilst nucleoprotein is increasing in the egg [Robertson and Wasteneys, 1913]. The same author, working with Burnett, has also described decrease in the rate of growth of carcinoma in rats, which he ascribes to the injection of lecithin into the tumor [1913]. Again, Brailsford Robertson has indicated by experiments on developing echinoderm eggs that lecithin is the possible auto-catalyst of growth [1913] and Halliburton [1909] has pointed out that the formation of choline or choline soaps by the splitting up of lecithin or other phospholipins may cause that alteration of surface tension around the nucleus which is the prelude to division.

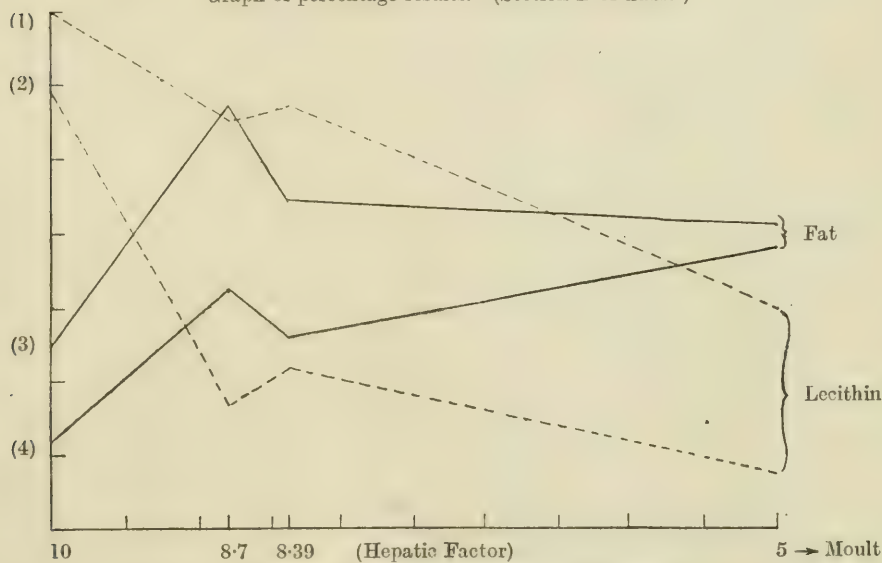
The present work therefore falls into line with previous researches. It shows that the phospholipin of crustacean livers decreases as cell proliferation advances, and the nuclei, though not the cell bodies, become larger. Since the integument is composed of calcium carbonate, the phosphorus cannot be associated with integument formation, comparable to calcium phosphate deposition in vertebrate bone. It is strongly probable, therefore, that at least some, if not all, of the phosphorus of the nucleoprotein molecule is derived from the phospholipin molecule.

Crab	A				B				
	Weight without legs	Weight of fresh liver	Fat in liver	Phospholipin as lecithin	Liver fat % of crab	Fat % of liver	Lecithin % of crab	Lecithin % of liver	Hepatic factor
1	283	53	10.95	0.077	3.8	20.6	0.03	0.7	5+
2	382	45.5	10.07	0.2179	2.6	22.1	0.057	2.1	8.39
3	339	38.7	11.01	0.1892	3.2	28.4	0.055	1.7	8.7
4	283	28.4	3.53	0.2091	1.2	12.4	0.07	5.9	10-

A=Quantitative results in grams.

B=Percentage results. These are graphically represented in relation to hepatic variation.

Graph of percentage results. (Section B of Table.)



- (1) Hepatic phospholipin percentage of crab.
- (2) Phospholipin percentage of fresh liver.
- (3) Fat percentage of fresh liver.
- (4) Hepatic fat percentage of crab.

On the Y-axis (1) Five small divisions = 0.01 %.
 (2) „ „ = 1 %.
 (3) „ „ = 5 %.
 (4) „ „ = 1 %.

We have pleasure in expressing indebtedness to Professor Noël Paton for his encouragement in the work, and for his sound advice on such parts of it as were carried out in the Physiological Department of Glasgow University.

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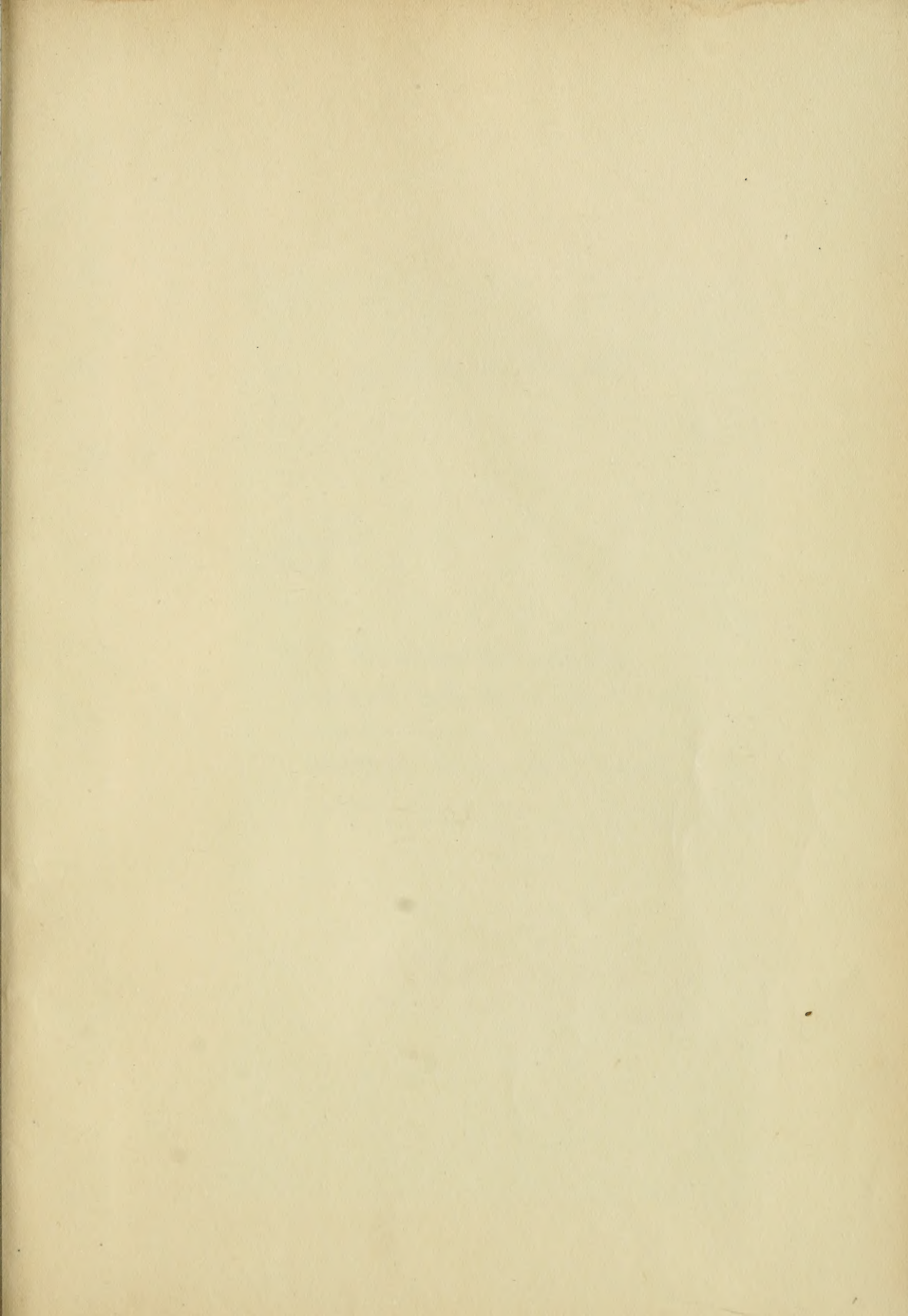
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INDEX

- Accessory food factors, action of ultra-violet rays on the (Zilva) 164
- Acetone, micro-estimation of, in blood (Widmark) 430
- Agglutinins, production of, effect of deficient nutrition on (Zilva) 172
- Albuminoid ammonia test (Cooper and Heward) 25
- Alcohol, effect of, on digestion of fibrin and caseinogen by trypsin (Edie) 219
- Amboceptor, production of, effect of deficient nutrition on (Zilva) 172
- Amino-acids (Dakin) 378
- Amino-acids, esters of, preparation of (Foreman) 378
- ANDREWES, F. A. Observations on the accuracy of different methods of measuring small volumes of fluid 37
- Antigenic specificity and chemical structure (Dakin and Dale) 248
- Antiscorbutic factor, rôle of, in nutrition (Drummond) 77
- Antiscorbutic value of dry and germinated seeds (Chick and Delf) 199
- Antiscorbutic value, relative, of fresh, dry and heated cow's milk (Barnes and Hume) 306
- Antitoxic sera, precipitation of, by sodium and ammonium sulphate (Homer) 278
- Antitoxin and associated proteins, separation of, from heat-denaturated sera (Homer) 45
- Antitoxin, associated with pseudoglobulin, increased precipitability of, from heat-denaturated solutions (Homer) 56
- Apple juice, composition of, effect of methods of extraction on (Haynes and Judd) 272
- Aspergillus niger*, mechanism of oxalic acid formation by (Raistrick and Clark) 329
- Bacillus coli*, effect of acids, alkalies and sugars on growth and indole formation of (Wyeth) 10
- Bacteria, cycloclastic power of (Raistrick) 446
- Bacteria, decomposition of histidine by (Raistrick) 446
- BARNES, R. E. and HUME, E. M. Relative antiscorbutic value of fresh, dry and heated cow's milk 306
- Blood, acetone in, micro-estimation of (Widmark) 430
- Blood pigment, nomenclature of (Halliburton and Rosenheim) 195
- Blood, sugar in, estimation of (MacLean) 135
- Blood, sugar in, estimation of, by picric acid method (de Wesselow) 148
- Box, C. R., *see* MELLANBY, J.
- BULLEY, E. C. Note on xerophthalmia in rats 103
- CAMPBELL, J. A. Nitrogen partition in the urine of the races in Singapore 239
- CANNAN, R. K., *see* HALLIBURTON, W. D.
- Caseinogen, composition of (Foreman) 378
- Caseinogen, digestion of, by trypsin, effect of alcohol on (Edie) 219
- CHICK, H. and DELF, E. M. The antiscorbutic value of dry and germinated seeds 199
- CLARK, A. B., *see* RAISTRICK, H.
- Cocoa butter, digestibility of (Gardner and Fox) 368
- Complement, production of, effect of deficient nutrition on (Zilva) 172
- COOPER, A. E., COOPER, E. A. and HEWARD, J. A. On the self-purification of rivers and streams 345
- COOPER, E. A. and HEWARD, J. A. Observations on the albuminoid ammonia test 25
- COOPER, E. A., *see also* COOPER, A. E.
- Crustacea, relationship of lecithin to growth cycle in (Paul and Sharpe) 487
- DAKIN, H. D. On amino-acids, Part II. Hydroxyglutamic acid 398
- DAKIN, H. D. and DALE, H. H. Chemical structure and antigenic specificity. A comparison of the crystalline egg-albumins of the hen and the duck 248
- DALE, H. H., *see* DAKIN, H. D.
- DELF, E. M., *see* CHICK, H.
- Diet, relation of sugar excretion to, in glycosuria (Mellanby and Box) 65
- Diffusion, rôle of plasma proteins in (Milroy and Donegan) 258
- Digestibility of cocoa butter (Gardner and Fox) 368
- DISTASO, A. and SUGDEN, J. H. Enterointoxication—its causes and treatment 153
- DONEGAN, J. F., *see* MILROY, T. H.
- DRUMMOND, J. C. Note on the rôle of the antiscorbutic factor in nutrition 77
- DRUMMOND, J. C. Researches on the fat-soluble accessory substance. I. Observations upon its nature and properties 81
- DRUMMOND, J. C. Researches on the fat-soluble accessory substance. II. Observations on its rôle in nutrition and influence on fat metabolism 95
- DRUMMOND, J. C., *see also* HALLIBURTON, W. D.
- Duck, egg-albumin of the, compared with that of the hen (Dakin and Dale) 248
- EDIE, E. S. The effect of alcohol on the digestion of fibrin and caseinogen by trypsin 219
- Egg-albumin, of the hen and duck, a comparison of (Dakin and Dale) 248

- Electrolytes of vegetable saps, electrical conductivity as a measure of the content of (Haynes) 111
- Enterointoxication, causes and treatment of (Distaso and Sugden)* 153
- Enzymes, oxidising, in plants (Onslow) 1
- Error, sampling, of fruit juices (Haynes and Judd) 272
- Fat metabolism, rôle of fat-soluble factor in (Drummond) 95
- Fat-soluble accessory substance, nature and properties of (Drummond) 81
- Fat-soluble accessory substance, rôle of, in nutrition and fat metabolism (Drummond) 95
- Fats, direct replacement of glycerol in, by higher polyhydric alcohols (Lapworth and Pearson) 296
(Halliburton, Drummond and Cannan) 301
- Fibrin, digestion of, by trypsin, effect of alcohol on (Edie) 219
- FISHER, E. R. Contributions to the study of the vegetable proteases 124
- FOREMAN, F. W. A new method for preparing esters of amino-acids. Composition of caseinogen 378
- FOX, F. W., *see* GARDNER, J. A.
- GARDNER, J. A. and FOX, F. W. On the digestibility of cocoa butter 368
- Gelatin sol, gelatin and hydrolysis of, velocity of (Shōji) 227
- Gelation of gelatin sol, velocity of (Shōji) 227
- Glycerol, direct replacement of, in fats by higher polyhydric alcohols
(Lapworth and Pearson) 296
(Halliburton, Drummond and Cannan) 301
- Glycosuria, relation of sugar excretion to diet in (Mellanby and Box) 65
- Growth of *B. coli*, effect of acids, alkalies and sugars on the (Wyeth) 10
- HALLIBURTON, W. D., DRUMMOND, J. C. and CANNAN, R. K. The direct replacement of glycerol in fats by higher polyhydric alcohols. Part II. The value of synthetic mannitol olive oil as a food 301
- HALLIBURTON, W. D. and ROSENHEIM, O. The nomenclature of blood pigment and its derivatives 195
- HAYNES, D. Electrical conductivity as a measure of the content of electrolytes of vegetable saps 111
- HAYNES, D. and JUDD, H. M. The effect of methods of extraction on the composition of expressed apple juice and a determination of the sampling error of such juices 272
- Hen, egg-albumin of the, compared with that of the duck (Dakin and Dale) 248
- HEWARD, J. A., *see* COOPER, A. E., and also COOPER, E. A.
- Histidine, bacterial decomposition of (Raistrick) 446
- HOMER, A. On the separation of antitoxin and its associated proteins from heat-denatured sera 45
- HOMER, A. On the increased precipitability of pseudoglobulin and of its associated antitoxin from heat-denatured solutions 56
- HOMER, A. A comparison between the precipitation of antitoxic sera by sodium sulphate and by ammonium sulphate 278
- HUME, E. M., *see* BARNES, R. E.
- Hydrolysis of gelatin sol, velocity of (Shōji) 227
- Hydroxyglutamic acid (Dakin) 398
- Indole formation of *B. coli*, effect of acids, alkalies and sugars on the (Wyeth) 10
- JUDD, H. M., *see* HAYNES, D.
- LAMPITT, L. H. Nitrogen metabolism in *Saccharomyces cerevisiae* 459
- LAPWORTH, A. and PEARSON, L. K. The direct replacement of glycerol in fats by higher polyhydric alcohols. Part I. Interaction of olein and stearin with mannitol 296
- Lecithin, relationship of, to growth cycle in crustacea (Paul and Sharpe) 487
- LEGG, A. T. The preparation of silica jelly for use as a bacteriological medium 107
- MACLEAN, H. On the estimation of sugar in blood 135
- Mannitol, interaction of, with olein and stearin (Lapworth and Pearson) 296
- Mannitol olive oil, synthetic, value of, as a food (Halliburton, Drummond and Cannan) 301
- MELLANBY, J. The composition of starch. Part I. Precipitation by colloidal iron. Part II. Precipitation by iodine and electrolytes 28
- MELLANBY, J. and BOX, C. R. The relation of sugar to diet in glycosuria 65
- Metabolism, fat, rôle of fat-soluble factor in (Drummond) 95
- Metabolism, nitrogen, of *S. cerevisiae* (Lampitt) 459
- Milk, cow's, fresh, dry and heated, relative antiscorbutic value of (Barnes and Hume) 306
- MILROY, T. H. and DONEGAN, J. F. The rôle of the plasma proteins in diffusion 258
- Nitrogen metabolism in *S. cerevisiae* (Lampitt) 459
- Nitrogen partition in urine of races in Singapore (Campbell) 239
- Nutrition, deficient, effect of, on production of agglutinins, complement and amboceptor (Zilva) 172
- Nutrition, rôle of antiscorbutic factor in (Drummond) 77
- Nutrition, rôle of fat-soluble factor in (Drummond) 95
- Olein, interaction of, with mannitol (Lapworth and Pearson) 296
- ONSLow, M. WHELDALe. Oxidising enzymes. I. The nature of the "peroxide" naturally associated with certain direct oxidising systems in plants 1
- Oxalic acid, mechanism of formation of, by *A. niger* (Raistrick and Clark) 329

- Oxidising enzymes in plants (Onslow) 1
- PAUL, J. H. and SHARPE, J. S. The relationship of lecithin to the growth cycle in crustacea 487
- PEARSON, L. K., *see* LAPWORTH, A.
- Peroxide, nature of, in oxidising systems in plants (Onslow) 1
- Picric acid method for estimation of sugar (de Wesselow) 148
- Plasma proteins, rôle of, in diffusion (Milroy and Donegan) 258
- Proteases, vegetable, the (Fisher) 124
- Proteins, of plasma, rôle of, in diffusion (Milroy and Donegan) 258
- Pseudoglobulin, increased precipitability of, from heat-denaturated solutions (Homer) 56
- RAISTRICK, H. Studies on the cycloclastic power of bacteria. Part I. A quantitative study of the aerobic decomposition of histidine by bacteria 446
- RAISTRICK, H. and CLARK, A. B. On the mechanism of oxalic acid formation by *Aspergillus niger* 329
- Rats, xerophthalmia in (Bulley) 103
- Rivers, self-purification of (Cooper, A. E. and E. A. and Heward) 345
- ROSENHEIM, O., *see* HALLIBURTON, W. D.
- Saccharomyces cerevisiae*, nitrogen metabolism in (Lampitt) 459
- Seeds, dry and germinated, antiscorbutic value of (Chick and Delf) 199
- Self-purification of rivers and streams (Cooper, A. E. and E. A. and Heward) 345
- Sera, antitoxic, precipitation of, by sodium and ammonium sulphate (Homer) 278
- Sera, heat-denaturated, separation of antitoxin and associated proteins from (Homer) 45
- SHARPE, J. S., *see* PAUL, J. H.
- SHÖJI, R. Studies on coagulation. I. On the velocity of gelatin and hydrolysis of gelatin sol 227
- Silica jelly, preparation of, for use as a bacteriological medium (Legg) 107
- Starch, composition of (Mellanby) 28
- Starch, precipitation of, by colloidal iron (Mellanby) 28
- Starch, precipitation of, by iodine and electrolytes (Mellanby) 28
- Stearin, interaction of, with mannitol (Lapworth and Pearson) 296
- Streams, self-purification of (Cooper, A. E. and E. A. and Heward) 345
- Sugar, estimation of, in blood (MacLean) 135
- Sugar, estimation of, in blood by picric acid method (de Wesselow) 148
- Sugar excretion, relation of, to diet in glycosuria (Mellanby and Box) 65
- SUGDEN, J. H., *see* DISTASO, A.
- Trypsin, digestion of fibrin and caseinogen by, effect of alcohol on (Edie) 219
- Ultra-violet rays, action of, on the accessory food factors (Zilva) 164
- Urine, nitrogen partition in, of races in Singapore (Campbell) 239
- Vegetable saps, electrolytes of (Haynes) 111
- Volumes of fluids, accuracy of measurement of small (Andrewes) 37
- WESSELOW, O. L. V. de. The picric acid method for the estimation of sugar in blood and a comparison of this method with that of MacLean 148
- WIDMARK, E. M. P. Studies in the acetone concentration in blood, urine, and alveolar air: I. A micromethod for the estimation of acetone in blood, based on the iodoform method 430
- WYETH, F. J. S. The effects of acids, alkalies and sugars on the growth and indole formation of *B. coli* 10
- Xerophthalmia in rats (Bulley) 103
- ZILVA, S. S. The action of ultra-violet rays on the accessory food factors 164
- ZILVA, S. S. The influence of deficient nutrition on the production of agglutinins, complement and amboceptor 172



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